mTOR COMPLEX 2 - AKT SIGNALING IS PHYSICALLY AND FUNCTIONALLY AT MAM

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CHARLES BETZ

aus LUXEMBURG

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On the infrequent occasions when I have been called upon in a formal place to play the bongo drums, the introducer never seems to find it necessary to mention that I also do theoretical physics.

— Richard Feynman
Messenger Lectures at Cornell University (1964-5)

Dedicated to my family

SUMMARY

The target of rapamycin (TOR) is a conserved protein kinase and a central controller of growth. TOR can be part of two structurally and functionally distinct complexes, termed TOR complex 1 and TOR complex 2. Mammalian TOR complex 2 (mTORC2) is composed of mTOR, Rictor, Sin1 and mLST8. Both mTORC1 and mTORC2 are activated by growth factors. The mechanism via which growth factors regulate mTORC2 has been elusive until recently. mTORC2 binds ribosomes in a growth factor stimulated manner and this association is required for mTORC2 activity.

mTOR complex 2 functions include control of spatial cell growth and metabolism and thus, mTORC2 deregulation has been linked to various disorders including cancer and diabetes. mTORC2 phosphorylates and thereby activates the AGC kinase family member Akt (PKB). Akt has many different targets and functions, not all of which depend on mTORC2 mediated Akt phosphorylation.

In order to gain a better understanding of mTORC2 function, we asked where mTORC2 signaling is localized. A number of studies localized mTORC2, functionally or physically, either to the endoplasmic reticulum (ER) or to mitochondria. We investigated whether these seemingly unrelated observations concerning mTORC2 localization, might be the consequence of mTORC2 signaling at MAM. MAM or mitochondria-associated ER membrane is a quasi-synaptic subdomain between the ER and mitochondria. MAM plays a crucial role in the regulation of mitochondrial metabolism and cell survival by gating both the calcium flux and phospholipid trafficking between the ER and mitochondria.

First, we analyzed mTORC2 subcellular localization. mTORC2 is localized to the ER adjacent to mitochondria and mTORC2 can be biochemically isolated from MAM structures. mTOR complex 2 interacts with the IP3R-Grp75-VDAC1 complex, a tether that connects ER and mitochondria at MAM. Insulin stimulates mTORC2 localization to MAM and mTORC2 interaction with the IP3R-Grp75-VDAC1 complex. MAM localization of mTORC2 depends on mTORC2-ribosome interaction.

Next we investigated the function of mTORC2 at MAM. Rictor (mTORC2) knockout causes a decrease in MAM formation. Growth factors stimulate MAM formation via mTORC2 and the Akt substrate PACS2, a MAM resident protein. As expected for MAM deficient cells, mTORC2 disruption changes the calcium flux from the ER to mitochondria at MAM. Furthermore, we observe a reduction of Akt mediated phosphorylation of the MAM calcium channel IP3R upon

Rictor knockout. Thus, mTORC2 signaling at MAM controls MAM-mediated calcium release via the Akt targets PACS2 and IP3R.

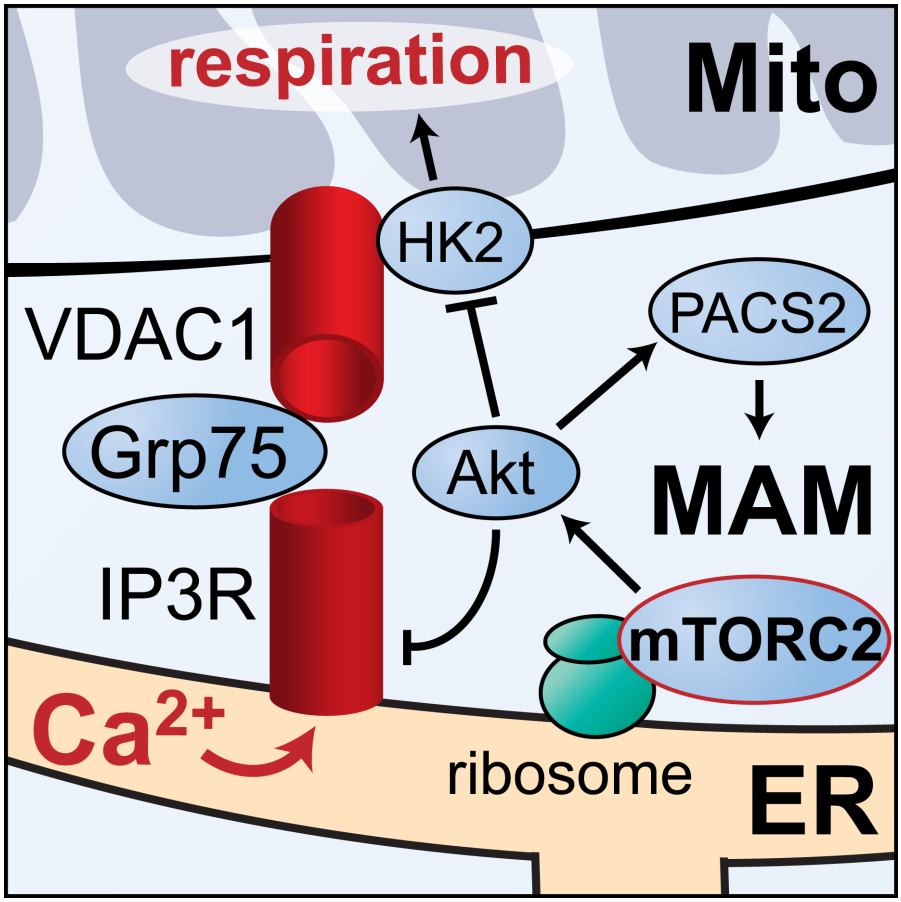
Since MAM disruption and calcium signaling both affect mitochondrial metabolism, we proceeded by analyzing the mitochondrial physiology of mTORC2 deficient cells. Rictor knockout cells exhibit a disruption of VDAC1-HK2 binding, caused by a lack of Akt mediated phosphorylation of HK2 at MAM. This, together with the defect in MAM, induces an increase in basal respiration, mitochondrial inner membrane potential, and ATP production in the mTORC2 deficient cells, culminating in apoptosis. Thus, mTORC2 at MAM appears to control several aspects of mitochondrial physiology.

These findings emphasize the role of MAM as a signaling hub that controls cell physiology. By identifying the integral role of mTORC2 at the core of this platform, our results provide new insights on the mechanisms that regulate growth and metabolism. These observations may offer new therapeutic strategies against mTORC2 and MAM driven diseases such as diabetes, Alzheimer's and cancer.

The results section of this thesis consists of the manuscript submitted to *Molecular Cell*. A number of complementing observations are presented in Section 2.2. The study characterizing the liver-specific knockout of Rictor, has important implications for my thesis and is presented as an appendix in Section 3.1.

Novel findings:

- mTORC2 localizes to MAM
- mTORC2 at MAM interacts with the IP3R-Grp75-VDAC1 complex and ribosomes
- mTORC2 at MAM phosphorylates Akt
- mTORC2 controls growth factor stimulated MAM formation via the Akt substrate PACS2
- mTORC2 controls MAM calcium release by regulating MAM integrity and the Akt substrate IP3R
- mTORC2 at MAM controls mitochondrial function via PACS2, IP3R and HK2
- MAM is an mTORC2-Akt signaling hub



Graphical abstract

And once the storm is over, you won't remember how you made it through, how you managed to survive. You won't even be sure, whether the storm is really over. But one thing is certain. When you come out of the storm, you won't be the same person who walked in. That's what this storm's all about.

—**Haruki Murakami**, *Kafka on the shore*

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I would like to thank all the people that have always encouraged me and that gave me the motivation and energy to give my best every day. First of all Line, who accepted all my countless over-hours and weekends in the lab, my parents for making me the person I am and my family for giving me the support to make it this far. My friends from all over the world, who despite their physical distance have always been close when I needed them.

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ACRONYMS

4**E-BP**1	Eukaryotic translation initiation factor 4E binding protein 1
ACSL4	Long-chain-fatty-acid-CoA ligase 4
AD	Alzheimer's disease
Ad-Cre	Fatty acid binding protein 4 promoter driven Cre recombinase
AdRicKO	Adipose-specific Rictor knockout mouse
AGC	Protein kinase A, G, and C families
Akt/PKB	Rac protein kinase alpha/ protein kinase B
AMPK	5' adenosine monophosphate-activated protein kinase
ATP	Adenosine triphosphate
AUC	Area under the curve
AU	Arbitrary units
BAT	Brown adipose tissue
Bip	Binding immunoglobulin protein, Grp78
BSA	Bovine serum albumin
Cer	Ceramide(s)
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate
CHX	Cycloheximide
CMV	Cytomegalovirus
CRIM	Conserved region in the middle, domain
DAVID	Database for Annotation, Visualization and Integrated Discovery
DEPTOR	DEP-domain-containing mTOR-interacting protein
DNA-PK	DNA-dependent protein kinase
ER	Endoplasmic reticulum
ERMES	ER-mitochondrion encounter structure
FAT	FRAP, ATM and TRRAP domain
FCS	Fetal calf serum
FIT	Found in TOR, domain
FKBP12	FK506-binding protein 12
FOXO	Forkhead box protein O
FRB	FKBP12 rapamycin binding domain
GILZ	Glucocorticoid-induced leucine-zipper protein
GK	Glucokinase
Grp75	Glucose regulated protein 75, HSPA9
Grp78	Glucose regulated protein 78, Bip

HCV	Hepatitis C virus
HeLa	Henrietta Lacks cell line
HK2	Hexokinase 2
HM	Hydrophobic motif
IMM	Inner mitochondrial membrane
IP	Immunoprecipitation
IP_3	Inositol trisphosphate
IP3R	Inositol trisphosphate receptor
IRES	Internal ribosome entry site
KO	Knockout (genetic)
LKB1	Liver kinase B1
MAM	Mitochondria associated ER membrane(s)
MAVS	Mitochondrial antiviral-signaling protein
Mfn	Mitofusin
mLST8	Mammalian lethal with SEC13 protein 8
MS	Mass spectrometry
mTOR	Mammalian [or mechanistic] target of rapamycin
mTORC1	mTOR complex 1
mTORC2	mTOR complex 2
NCL	Neuronal ceroid lipofuscinosis
NDGR1	N-myc downstream regulated
NF2	Neurofibromin 2
NGF1	Nerve growth factor I
OMM	Outer mitochondrial membrane
PA	Phosphatidic acid
PACS2	Phosphofurin acidic cluster sorting protein 2
PAM	Plasma membrane associated ER membrane(s)
PDK1	Phosphoinositide-dependent kinase-1
PE	phosphatidylethanolamine
PEMT2	Phosphatidylethanolamine N- methyltransferase 2
PEPCK	Phosphoenolpyruvate carboxykinase
PG	Phosphatidylglyerol
PH	Pleckstrin homology domain
PI3K	Phosphatidylinositol 3-kinase
PI3P	Phosphatidylinositol 3-phosphate
PIKK	Phosphatidylinositol kinase-related kinases
PINK1	PTEN-induced putative kinase 1
PKA	Protein kinase A

PKC	Protein kinase C
PLD	Phospholipase D
PML	Promyelocytic leukemia protein
PPARγ	Peroxisome proliferator-activated receptor gamma
PRAS40	Proline-rich Akt/protein kinase B [PKB] substrate 40 kDa
PRR5	Proline-rich protein 5 [also called Protor]
PS	Phosphatidylserine
PSS1	Phosphatidylserine Synthase-1
PTEN	Phosphatase and tensin homolog deleted on chromosome ten
PTP	Permeability transition pore
qPCR	Quantitative Real-time polymerase chain reaction
Raptor	Regulatory associated protein of mTOR complex 1
RBD	Ras binding domain
RER	Rough endoplasmic reticulum
Rictor	Rapamycin insensitive companion of mTOR
Rpl26	Large ribosomal subunit protein 26
S6**K**	Ribosomal protein S6 kinase
SER	Smooth endoplasmic reticulum
SGK1	Serum/glucocorticoid regulated kinase 1
Sig-1**R**	Sigma 1 receptor
Sin1	SAPK [stress-activated protein kinase]-interacting protein 1
SREBP-1	Sterol regulatory element binding protein-1
SRP	Signal recognition particle
TCA	Tricarboxylic acid cycle or citric acid cycle
TCP1	T-complex protein 1 subunit alpha
TGFβ	Transforming growth factor beta
TH1	T helper cell 1
TLC	Thin layer chromatography
TMX	Thioredoxin-related protein
TNFα	Tumor necrosis factor alpha
TOS	TOR signaling motif
TSC	Tuberous sclerosis complex
ULK1	Unc-51-like kinase 1
UTR	Untranslated region
VDAC1	Voltage-dependent anion channel
WT	genetic wildtype or control as opposed to KO

1 INTRODUCTION

1.1 TOR SIGNALING

1.1.1 *Discovery and history of TOR*

1.1.1.1 *Rapamycin*

The history of TOR research begins in 1965 on Rapa Nui, better known as Easter Island. A research team found that the common bacterium *Streptomyces hygroscopicus* isolated from one of their soil samples, secreted a macrolide that inhibits growth of the fungus *Candida albicans*, and, accordingly, they named this novel antibiotic rapamycin (Vézina et al., 1975). Importantly, rapamycin was subsequently found to also have immunosuppressive and anti-proliferative properties in mammalian cells. Over 20 years later, rapamycin was approved for use in post-transplantational immunosuppression (Camardo, 2003).

1.1.1.2 *Discovery of TOR*

The growth inhibitory effect of rapamycin spiked an interest in identifying its target protein. *Saccharomyces cerevisiae* is an ideal model system to perform genetic screens to identify rapamycin resistant mutants. In 1991, a study identified three genes in yeast that upon loss confer rapamycin resistance (Heitman et al., 1991). One of these genes was the immunophillin FKBP12 (FK506 binding protein 12, a prolyl isomerase). FKBP12 was at the time already known to mediate the effect of FK506 (another immunosuppressant structurally related to rapamycin) toward calcineurin. FKBP12 is highly conserved among eukaryotes, with the exception of *A. thaliana* and *C. elegans*, both of which lack a rapamycin binding FKBP12 homolog and thus are rapamycin resistant.

Another class of mutations was found in two related genes termed TOR1 (target of rapamycin 1) and TOR2 (Heitman et al., 1991). This important discovery marks the foundation of TOR research and subsequent investigations revealed that TOR is part of two protein complexes, only one of which is targeted allosterically by the rapamycin-FKBP12 complex. Both the use of the specific inhibitor rapamycin and the combination of biochemistry and genetics in yeast greatly facilitated the subsequent research on the two TOR complexes (Loewith et al., 2002).

1.1.1.3 *Conservation of TOR*

TOR is structurally and functionally conserved throughout eukaryotic evolution, as the effect of rapamycin on both yeast and mammalian cells had already suggested. Mammalian TOR (mTOR) was discovered in 1994 and found to control growth in a similar manner as in yeast (Brown et al., 1994; Sabatini et al., 1994; Chiu et al., 1994; Sabers et al., 1995). In yeast, nutrient (i.e. nitrogen and amino acids) availability is relayed to TOR (Barbet et al., 1996; Hara et al., 1998; X Wang, 1998). Multicellular organisms, presenting a further dimension of growth control, also feed growth factor signaling to TOR, in order to control and synchronize whole body growth and metabolism. The following parts will mostly address the single mammalian TOR gene, called mTOR.

A selection of recent, TOR-related reviews

Laplante and Sabatini (2012)	mTOR in growth and disease
Appenzeller-Herzog and Hall (2012)	mTOR and the ER
Durán and Hall (2012)	TOR and GTPases
Oh and Jacinto (2011)	mTORC2 review
Efeyan and Sabatini (2010)	mTOR and cancer
Pearce et al. (2010)	AGC kinases
Laplante and Sabatini (2009b)	mTOR and lipid biosynthesis
Cybulski and Hall (2009)	mTORC2 review
Soulard et al. (2009)	TOR signaling in invertebrates
Blagosklonny and Hall (2009)	Growth and aging

1.1.2 *Organization of domains in TOR*

TOR is the founding member of a kinase family called PIKK (phosphoinositide kinase-related kinase) family that comprises ATM, ATR, DNA-PK, SMG1, TRRAP and TOR. All members of this atypical kinase family are protein kinases, even though they are structurally related to phosphoinositide 3-kinases (PI3Ks) and PI4Ks which are lipid kinases (Benjamin et al., 2011). The PIKKs share similar domain organization with a $\pm$ 600 residue helical domain (FAT) and a shorter ($\pm$ 70 residues), similar domain at the C-terminus (FATC). Only TOR contains a FRB domain ($\pm$ 100 residues) between the helical FAT and the catalytic kinase domain ($\pm$ 370 residues). FRB, the FKBP12-rapamycin binding domain, is presumably masked in TORC2 by one of the specific subunits (Hall, 2008). A large part of the N-terminal part of TOR is formed by roughly 20 HEAT (huntingtin, elongation factor 3, PP2A and TOR) repeats, a domain that most likely forms an extended superhelical structure (Groves, 1999) and that plays a role

in protein-protein interaction with Raptor, one of the mTOR complex 1 (mTORC1) subunits (Kim et al., 2002). In between the catalytic domain and the FATC domain is the FIT domain (Found In TOR) that in mTOR encompasses several phosphorylation sites (Sturgill and Hall, 2009).

The TOR complexes most likely exist as dimers. mTORC1 dimerization is nutrient- but not growth factor-responsive (Zhang et al., 2006b; Takahara et al., 2006) although the role of this organization is not yet known (Wullschleger et al., 2005; Yip et al., 2010). One hypothesis is that dimerization of the mTOR complex facilitates intramolecular phosphorylation. Currently, only a low resolution 3D structure model of mTORC1 is published. This model comes from cryo-electron microscopic images and shows mTORC1 as a dimer (Yip et al., 2010).

Interestingly, there is a splice variant of mTOR, called mTORβ (Panasyuk et al., 2009). It is a short isoform and in comparison to full length mTOR, mTORβ induces higher S6K and Akt phosphorylation when overexpressed. mTORβ is believed to be tumorigenic.

1.1.3 *Components of the mTOR complexes*

1.1.3.1 *mTORC1*

mTOR complex 1 is composed of mTOR, Raptor and mLST8. Raptor (Kog1 in yeast) plays an essential role in mTORC1 by stabilizing the complex (Kim and Sabatini, 2004) and by recruiting substrates to mTOR via interaction of its RNC (Raptor N-terminal conserved) domain with the TOS motif found in some TOR substrates (Nojima et al., 2003; Schalm et al., 2003; Adami et al., 2007). mLST8 (mammalian lethal with sec thirteen 8) is a small protein composed of 7 WD40 repeats that is found both in mTORC1 and mTORC2. mLST8 enhances mTOR kinase activity of mTORC1 (Kim et al., 2003; Wullschleger et al., 2006). However, mLST8 presence is required for mTORC2 but not mTORC1 activity (Guertin et al., 2006; Wang et al., 2012).

PRAS40 (AKT1S1) can associate with mTORC1 and acts as a negative regulator towards mTORC1 activity (Sancak et al., 2007; Vander Haar et al., 2007). Deptor can interact with both mTOR complexes and can regulate mTOR activity both positively and negatively (Peterson et al., 2009). Deptor contains two DEP domains (disheveled, egl-10, pleckstrin) and a PDZ (postsynaptic density 95, discs large, zonula occludens-1) domain. Deptor expression and translation are regulated by mTORC1 and mTORC2. Both PRAS40 and Deptor are not considered to be integral mTOR complex components.

1.1.3.2 *mTORC2*

mTOR complex 2 is composed of mTOR, Rictor, Sin1 and mLST8. Rictor is homologous to Avo3 in yeast (25% identity) (Jacinto et al.,

2004). No obvious domains can be identified in Rictor, however the C-terminal part is highly conserved in vertebrates. Sin1 is homologous to Avo1 in yeast (Frias et al., 2006; Jacinto et al., 2006). Sin1 undergoes alternative splicing and gives rise to 5 different isoforms, termed Sin1.1-Sin1.5. Sin1.1, Sin1.2 and Sin1.5 can be part of mTORC2, while Sin1.3 is not expressed in HeLa cells and was not further studied (Cheng et al., 2005; Frias et al., 2006). Sin1.1 and Sin1.2 both have a pleckstrin homology (PH) domain that presumably associates them with membranes. Other domains in Sin1 are the conserved region in the middle (CRIM) and the ras-binding domain (RBD). Among the mTORC2 binding Sin1 isoforms, RBD is only found in Sin1.1. The RBD domain was found to bind and activate Ras when overexpressed (Schroder et al., 2007). Sin1.5 might be part of an insulin insensitive mTORC2 (Frias et al., 2006). The different Sin1 isoforms suggest the existence of at least 3 different mTORC2s. It is unknown which role these different complexes play in mTORC2 signaling. Sin1 furthermore has mTORC2 independent functions in the stress response (Cheng et al., 2005; Schroder et al., 2007; Makino et al., 2006; Schroder et al., 2005; Ghosh et al., 2008). Rictor and Sin1 stabilize each other, as deletion of one can affect the levels of the other (Jacinto et al., 2006; Guertin et al., 2006; Yang et al., 2006). mLST8 deletion inhibits association of Rictor with mTOR but not association of Raptor with mTOR or that of Rictor with Sin1 (Guertin et al., 2006; García-Martínez and Alessi, 2008).

Another class of mTORC2 interacting proteins is PRR5/PRR5L (Protor 1/2) that bear minimal sequence homologies to the yeast TORC2 component Bit61 (Pearce et al., 2007; Woo et al., 2007; Thedieck et al., 2007). PRR5/PRR5L are the only non essential mTORC2 components. PRR5 is in a complex with Rictor even when mTORC2 is disrupted. Rictor regulates PRR5 expression and PRR5 is overexpressed in certain cancers (Johnstone et al., 2005; Oh and Jacinto, 2011).

Other less well studied factors interact with mTORC2 but should not be considered as components of the complex. Hsp70 binds Rictor and depletion of Hsp70 affects mTORC2 activity (Martin et al., 2008). Tel2 and Tti play a role in mTOR complex formation and stability (Takai et al., 2007; Kaizuka et al., 2010).

1.1.4 *Regulation of the mTOR complexes*

1.1.4.1 *Upstream regulation of mTORC1*

Because of the availability of specific mTORC1 inhibitors, upstream signaling of mTORC1 is generally better understood than that of mTORC2. mTORC1 can be posttranslationaly modified. mTOR, primarily in mTORC1 but also in mTORC2 is phosphorylated at S2448. However it is not clear whether this phosphorylation affects mTORC1 activity.

Deptor, as mentioned above, associates with both mTOR complexes. Its function is not entirely clear, but it might negatively regulate their activity under some conditions, while acting positively in others (Peterson et al., 2009). Interestingly, Deptor expression and translation are negatively regulated by the two mTOR complexes. It should be noted however that the logic and mechanism of this regulatory loop are not well understood at this moment.

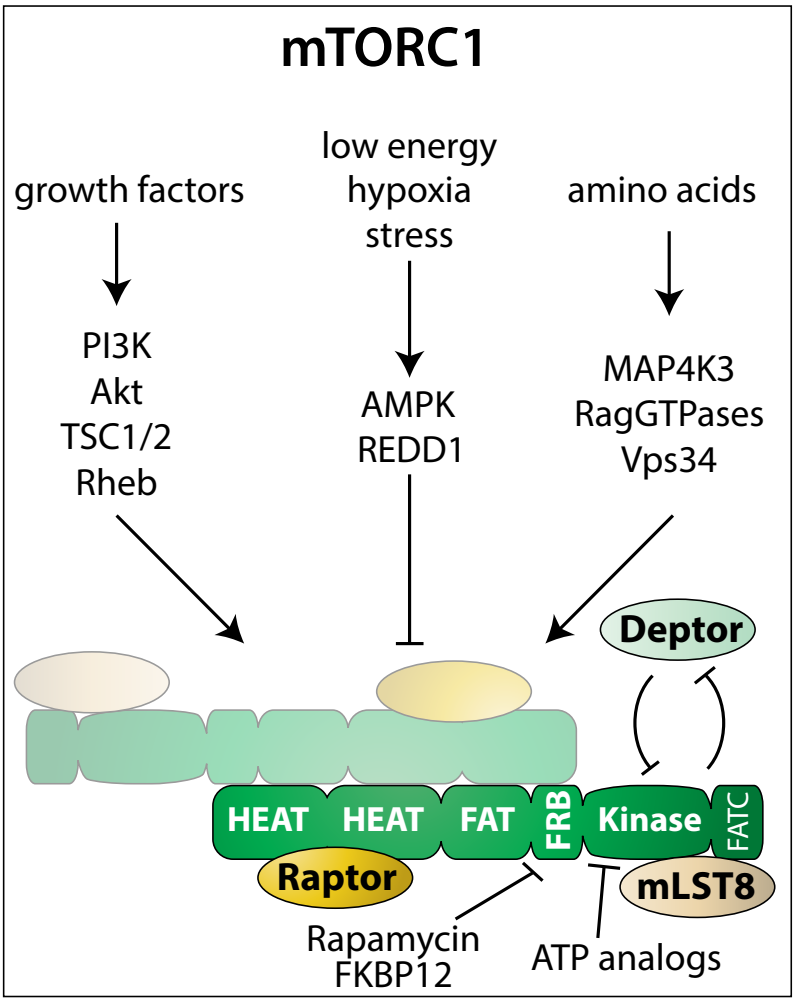

Figure 1: **mTORC1 regulation**

NUTRIENTS Amino acids are necessary for mTORC1 activity (Hara et al., 1998; X Wang, 1998). The exact sensing mechanism is only partially understood and includes MAP4K3 (Findlay et al., 2007; Yan et al., 2010), VPS34 (Gulati et al., 2008; Nobukuni et al., 2005) and the Rag GTPases (Sancak et al., 2008; Kim et al., 2008). Upon amino acid availability, Raptor interacts with the RagA/RagB RagC/RagD heterodimer, associating mTORC1 with the lysosomal surface. At the lysosome, GTP-bound Rheb (stimulated by growth factors), activates TOR. Rag activity depends on GTP/GDP loading state and one of the mechanisms by which leucine and glutamine activate Rags, is through glutaminolysis (Durán et al., 2012).

GROWTH FACTORS Insulin and other growth factors activate PI3K. PI3K phosphorylates PIP_2 to form PIP_3 in the plasma membrane. Akt, via its PH domain, can bind to PIP_3. Akt is then activated by PDK1-mediated phosphorylation at T308. Akt in turn phosphorylates TSC2, thereby inhibiting the GAP activity of the TSC1/TSC2 complex and favoring Rheb-GTP loading (Inoki et al., 2002; Manning et al., 2002; Potter et al., 2002; Garami et al., 2003; Inoki et al., 2003; Tee et al., 2003). A GEF for Rheb is currently unknown. As mentioned earlier, Rheb-GTP at the lysosome then activates mTORC1 if amino acids are present. Accordingly, Rheb is responsible for both amino acid and growth factor mediated activation of mTORC1. Thus, overexpression of Rheb rescues mTORC1 activity even under absence of growth factors or amino acids.

Akt also regulates mTORC1 by phosphorylating PRAS40, thereby sequestering it away from mTORC1 and alleviating the inhibitory role of PRAS40 toward mTORC1 (Kovacina et al., 2003). Further signaling inputs from growth factors come from Erk and GSK3β phosphorylation of TSC2 (Ma et al., 2005; Castilho et al., 2009; Inoki et al., 2006).

ENERGY mTOR complex 1 senses the cellular energy status through AMPK (Hardie, 2007). If the AMP:ATP ratio rises, AMPK phosphorylates TSC2, and this presumably stimulates TSC GAP activity (Corradetti et al., 2004). AMPK also phosphorylates and thereby inhibits Raptor (Gwinn et al., 2008). This arm of mTORC1 signaling guarantees mTORC1 inhibition when energy levels are low.

STRESS Some forms of stress such as hypoxia also act through the above mentioned AMPK-mTORC1 route. Hypoxia furthermore reduces mTORC1 activity through REDD1 (Zoncu et al., 2010). DNA damage inhibits mTORC1 via p53-AMPK (Feng et al., 2007; Jones et al., 2005)

1.1.4.2 *Upstream regulation of mTORC2*

mTORC2 is stimulated by growth factors. Growth factors, via PI3K, induce mTORC2-ribosome association (Oh et al., 2010; Zinzalla et al., 2011). Active mTORC2 binds to the large ribosomal subunit, independently of translation. Ribosome perturbation leads to a loss of TORC2 activity in yeast and mammals, even though growth factor signaling has been grafted onto TORC2 signaling at an evolutionary later step. The exact mechanism of how ribosome binding might influence mTORC2 activity is unknown. One hypothesis is that the ribosome anchors mTORC2 inside the cell at a localization that activates mTORC2.

mTORC2 is generally thought not to be regulated by nutrients, however there are conflicting observations regarding mTORC2 activation by amino acids (Tato et al., 2011; Nobukuni et al., 2005; Hernández-Negrete et al., 2007).

Several posttranslational modifications affect mTORC2 activity directly. Rictor can be acetylated, a modification that stabilizes mTORC2 (Glidden et al., 2012). mTOR in mTORC2 can be autophosphorylated at S2481, and this phosphorylation might be a marker for mTORC2 rapamycin sensitivity (Copp et al., 2009). S2481 is also sensitive to PI3K inhibition (Peterson et al., 2000). Nevertheless, the functional consequence of this phosphorylation is not yet understood (Copp et al., 2009; Soliman et al., 2010). Rictor and Sin1 are phosphorylated on multiple sites (Akcakanat et al., 2007; Dibble et al., 2009). For example, Rictor is phosphorylated at T1135 by S6K1. However, conflicting observations were reported as to whether this phosphorylation is inhibitory (Julien et al., 2010; Treins et al., 2010; Boulbes et al., 2010). Furthermore, Rictor is phosphorylated at S1235 by GSK3β in response to ER stress (Chen et al., 2011). There might be alternative ways by which ER stress inhibits mTORC2 signaling (Appenzeller-Herzog and Hall, 2012). Sin1 phosphorylation enhances its binding to mTOR but does not affect Sin1 binding to Rictor (Yang et al., 2006; Rosner and Hengstschläger, 2008).

mTORC2 activity is enhanced by PIP_3 directly (Gan et al., 2011). This apparent activation might be due to a conformational change in the PH domain of the mTORC2 substrate Akt, facilitating mTORC2 access to the hydrophobic motif.

mTORC2 activity is upregulated upon inhibition of Epac1, thus possibly putting cAMP signaling upstream of mTORC2 (Misra and Pizzo, 2012). Furthermore, mTORC2 might be involved in Notch signaling, as deletion of Rictor in T-cells ablated Notch driven processes (Lee et al., 2012). mTORC2 activity is reduced in NF2 (neurofibromin 2) deficient cells (James et al., 2012). NF2 or merlin is the tumor suppressor lost in neurofibromatosis type 2, a non-malignant, inherited cancer disease. TGFβ impinges on mTORC2 activity in the context of epithelial to mesenchymal transition (Lamouille et al., 2012).

The glucocorticoid-induced leucine zipper protein (GILZ) can bind mTORC2 and inhibit its activity (Joha et al., 2011). This suggests that glucocorticoid treatment might be beneficial in cancer therapeutics by inhibiting mTORC2. The TSC complex might bind mTORC2 leading to mTORC2 activation (Huang and Manning, 2009; Huang et al., 2008), however this finding has been questioned by other studies (Dalle Pezze et al., 2012). mTORC2 signaling is inhibited in Syndecan-4 null endothelial cells, possibly due to a reduced amount of mTORC2 components associated with lipid rafts (Partovian et al., 2008).

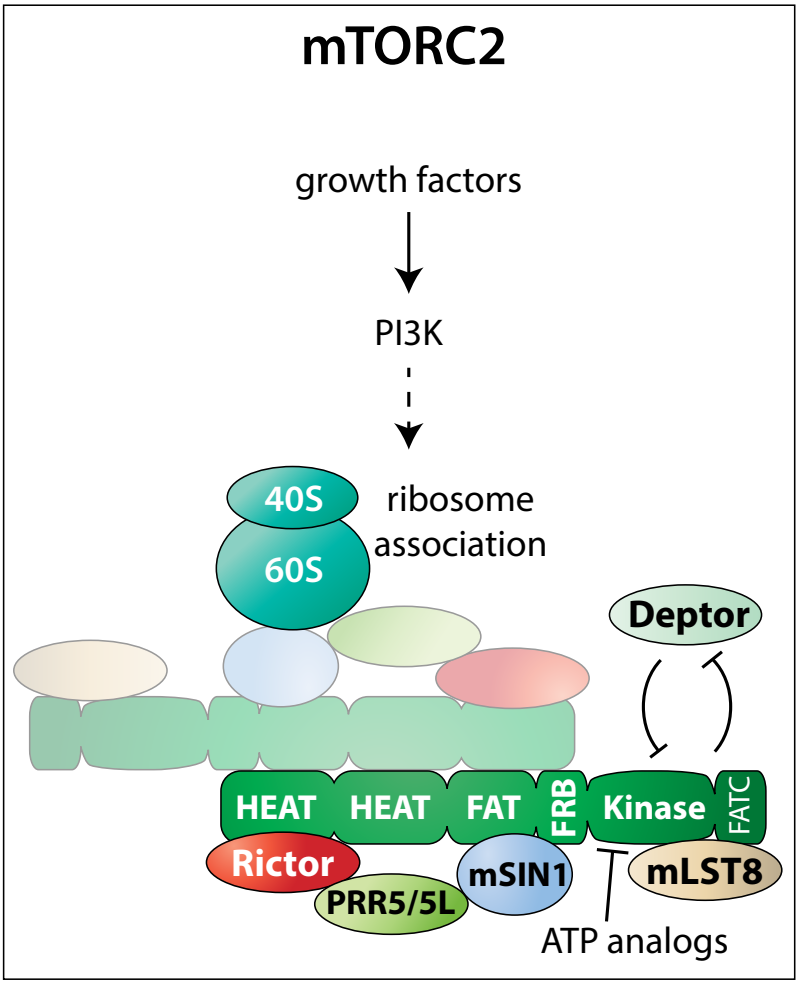

Figure 2: **mTORC2 regulation**

While mTORC2 is generally thought to be insensitive to rapamycin, there are some cell lines that show reduced mTORC2 activity upon rapamycin treatment. Long-term rapamycin treatment is thought to sequester free mTOR away as mTOR-FKBP12-rapamycin. Since rapamycin inhibits mTORC1 and thereby translation, synthesis of new mTOR complex components is reduced and thus, mTORC2 formation is inhibited (Sarbassov et al., 2006). Inhibition of phospholipase D (PLD) activity increases the sensitivity of mTORC2 to rapamycin,

since phosphatidic acid (PA), the metabolite of PLD, can compete with rapamycin for FRB binding (Toschi et al., 2009; Fang et al., 2001).

A coherent model of mTORC2 upstream signaling that integrates these unconnected observations, is currently missing. mTORC2 signaling in general is less studied than mTORC1 signaling, possibly due to the lack of specific inhibitors. It is also conceivable that mTORC2 regulation is more cell type specific than that of mTORC1, and thus more difficult to delineate.

1.1.5 *Substrates of the mTOR complexes*

The majority of mTOR targets are AGC kinase family members such as Akt, p70 ribosomal kinase 1 (S6K1), SGK1 and PKC. mTOR phosphorylates these targets at a so-called hydrophobic motif (HM), that consists of Phe-X-X-Phe-Ser/Thr-Tyr, and the specific mTOR complex components (Raptor, Sin1 and Rictor) might be important for recruiting and presenting the targets to mTOR.

1.1.5.1 *mTORC1 targets and function*

S6K1 Ribosomal protein S6 kinase β-1 (S6K1) promotes translation initiation and elongation via multiple proteins including eEF3K, SKAR, CBP80 and eIF4B (Zoncu et al., 2010). Furthermore, mTORC1 via S6K regulates ribosome biogenesis by controlling RNAPI (Mayer et al., 2004). This couples the energy status of the cell, which is sensed by mTORC1, to the regulation of cell growth via ribosome biogenesis. Dietary restriction prolongs life span by inhibiting mTOR complex 1. S6K1 is implicated in aging and might be the main target in mTORC1 mediated life span regulation (Selman et al., 2009).

S6K1 also mediates a negative feedback loop in mTORC1 signaling. S6K phosphorylates and thereby inhibits IRS1, which in turn dampens the response of the PI3K pathway to growth factors. This leads to insulin resistance under chronically high mTORC1 signaling (Um et al., 2004; Shah et al., 2004; Harrington et al., 2004). Conversely, inhibition of the negative feedback loop leads to activation of Akt and thereby triggers a pro-survival response (O'Reilly et al., 2006). The negative feedback loop is thought to limit the potential of mTORC1 inhibition in cancer therapeutics.

4E-BP1 eIF4E-binding protein (4E-BP1) associates with mRNAs in order to control translation initiation and elongation (Ma and Blenis, 2009). Upon phosphorylation by mTORC1, 4E-BP1 dissociates from eIF4E, thus allowing eIF4G recruitment to the 5′ end of the message. 4E-BP1 mediates mTORC1 regulation of proliferation, while S6K1 mediates mTORC1 regulation of cell size (Dowling et al., 2010).

AUTOPHAGY mTORC1 suppresses macroautophagy through phosphorylation of ATG13 and ULK1, effectively blocking autophagosome formation (Hosokawa et al., 2009).

OTHER TARGETS mTORC1 regulates several transcription factors. Among them, SREBP and PPARγ control lipid synthesis (Kim and Chen, 2004; Porstmann et al., 2008) while PGC1α and YY1 control mitochondrial biogenesis and function (Cunningham et al., 2007).

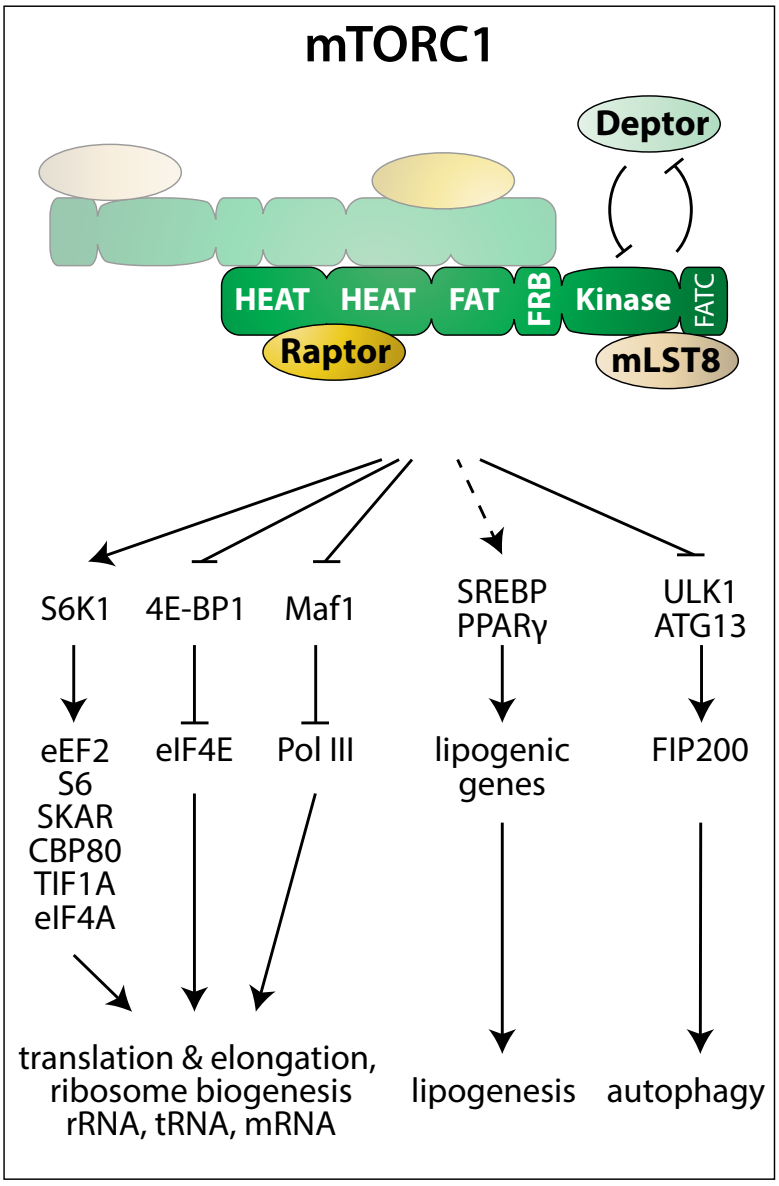

Figure 3: **mTORC1 targets** (Laplante and Sabatini, 2012)

1.1.5.2 *mTORC2 targets and function*

mTORC2 regulates spatial cell growth and actin organization. This function of TORC2 is conserved from yeast to mammals (Schmidt et al., 1996; Jacinto et al., 2004; Sarbassov et al., 2004) and led to an increased interest in mTORC2 because of its potential role in tumor cell metastasis and invasion (Zhou and Huang, 2011). The GTPase Rac1 that binds mTORC2 is possibly involved in regulating the cytoskeleton (Saci et al., 2011). The final verdict on how mTORC2 regulates cytoskeleton organization is far from clear. For example, loss of mTORC2 has been attributed to both an increase or decrease of actin stress fiber formation (Jacinto et al., 2004; Sarbassov et al., 2004). Furthermore, MEFs from Rictor knockout mice failed to show any obvious alterations in actin organization (Guertin et al., 2006; Shiota et al., 2006). This discrepancy might come from an adaptation in the regulation of the cytoskeleton upon long-term mTORC2 inhibition (Cybulski and Hall, 2009).

Results from *C. elegans* and tissue-specific knock-out mice indicate an important involvement of TORC2 in the metabolism of specific tissues and of the whole body (Gu et al., 2011; Cybulski et al., 2009; Soukas et al., 2009).

AKT mTOR complex 2 in mammalian cells directly phosphorylates Akt on its hydrophobic motif, S473 (Sarbassov et al., 2005). Phosphorylation of Akt by mTORC2 at S473 in combination with phosphorylation of Akt by PDK1 in the activation loop at T308 leads to full Akt activation. Phosphorylation of T308 by PDK1 in Akt does not depend on prior phosphorylation by mTORC2. mTORC2 might also phosphorylate the turn motif in Akt at T450 (Facchinetti et al., 2008; Ikenoue et al., 2008). Interestingly, this phosphorylation, unlike mTORC2 activity, is not regulated by growth factors. T450 in Akt is phosphorylated co-translationally by ribosome-associated mTORC2 and is important for Akt stability (Oh et al., 2010). However, it is not clear why mTORC2 association to the ribosome is growth factor regulated, while phosphorylation at T450 is not. Furthermore, there are controversial results concerning direct phosphorylation of Akt at the turn motif by mTORC2 *in vitro* (Alessi et al., 2009). An alternative hypothesis as to how mTORC2 regulates turn motif phosphorylation is that mTORC2 might control a phosphatase dephosphorylating this site.

Loss of mTORC2 activity prevents phosphorylation of some, but not all Akt targets (Yang et al., 2006; Guertin et al., 2006; Soukas et al., 2009). For example, TSC2 phosphorylation is not changed upon mTORC2 knockout. This suggests that mTORC2 activated Akt is not upstream of mTORC1, thus indicating different pools of Akt inside the cell. S473 phosphorylation of Akt might regulate Akt substrate specificity rather than absolute activity, thus explaining why not all

Akt targets are affected by the loss of mTORC2. There is some discrepancy as to whether S473 affects the T308 branch of Akt signaling. A reduction in T308 phosphorylation was reported upon loss of mTORC2 (Yang et al., 2006), however later studies could not confirm this observation (Jacinto et al., 2006). It was suggested that in cancer cells, for example upon loss of PTEN, T308 and S473 phosphorylation on Akt might become linked (Guertin et al., 2009).

mTORC2 controls glycogen accumulation via Akt mediated phosphorylation of GSK3β (Cross et al., 1995). GSK3β phosphorylation however is not reduced in all cell types upon knockout of mTORC2. A possible explanation for this comes from the fact that GSK3β can also be phosphorylated by other kinases such as S6K (Zhang et al., 2006a).

A more consistently mTORC2-regulated Akt target is the transcription factor FOXO1. mTORC2 activated Akt phosphorylates FOXO1, and thereby inhibits it. FOXO1 affects expression of many genes, one of which is the Toll-like receptor 4. This might be one way by which mTORC2 regulates inflammation (Brown et al., 2011). Another target of FOXO1 is PEPCK, thus making mTORC2 a negative regulator of gluconeogenesis (Puigserver et al., 2003). Interestingly, FOXO1 can also be phosphorylated by SGK1, another direct mTORC2 target (Brunet et al., 2001), thus making FOXO1 phosphorylation a robust readout for mTORC2 activity.

Other mTORC2 - Akt targets include Bad (proapoptotic) and AS160 (GLUT4 trafficking) (Brazil et al., 2004; Datta et al., 1997; Kumar et al., 2010). Phosphorylation of Bad by Akt results in the dissociation of Bad/Bcl-2 complexes and hence the inhibition of apoptosis. Furthermore, Akt can phosphorylate Bax directly, thereby inhibiting caspase 3 release (Gardai et al., 2004).

SGK1 mTORC2 directly phosphorylates SGK1 (García-Martínez and Alessi, 2008). This event regulates SGK1 activity toward NDRG1. Experiments in yeast and worms suggest that SGK1 might be the most important direct downstream target of mTORC2, however it is not clear yet whether this is also the case in mammalian cells. SGK1 shares some targets with Akt, such as FOXO and GSK3β, but also possesses unique targets such as Nedd4 − 2, an ubiquitin E3 ligase (Alessi et al., 2009). Phosphorylation by SGK1 sequesters Nedd4 − 2 away from ENaC, the epithelial sodium channel, thus enhancing sodium transport (Debonneville et al., 2001; Ichimura et al., 2005; Loffing et al., 2006).

Unlike Akt, SGK1 activity is completely abolished if mTORC2 is disrupted. Thus, if mTORC2 is knocked out, all SGK1 targets are fully inhibited, while only a subset of Akt targets is inactive (Jacinto et al., 2006; Guertin et al., 2006).

PKC mTORC2 phosphorylates all conventional PKC family members and PKCε directly at the hydrophobic motif (Ikenoue et al., 2008; Facchinetti et al., 2008). This phosphorylation controls PKC activity and stability. The PKC family members diverge at their N-terminal regulatory region. They do however have overlapping substrates (Newton, 2010). The cytoskeletal defects in mTORC2 knockout cells might be partially due to reduced PKC signaling. To this date, no physiological signals that regulate PKC phosphorylation via mTORC2 are known. Furthermore, it is currently unclear which proteins mTOR complex 2 controls via PKC. Results from PKC and Akt knockout mice suggest that Akt is probably a physiologically more relevant mTORC2 target than PKC (Grahammer et al., 2006; Peng et al., 2003; Yang et al., 2005; Chen et al., 2006).

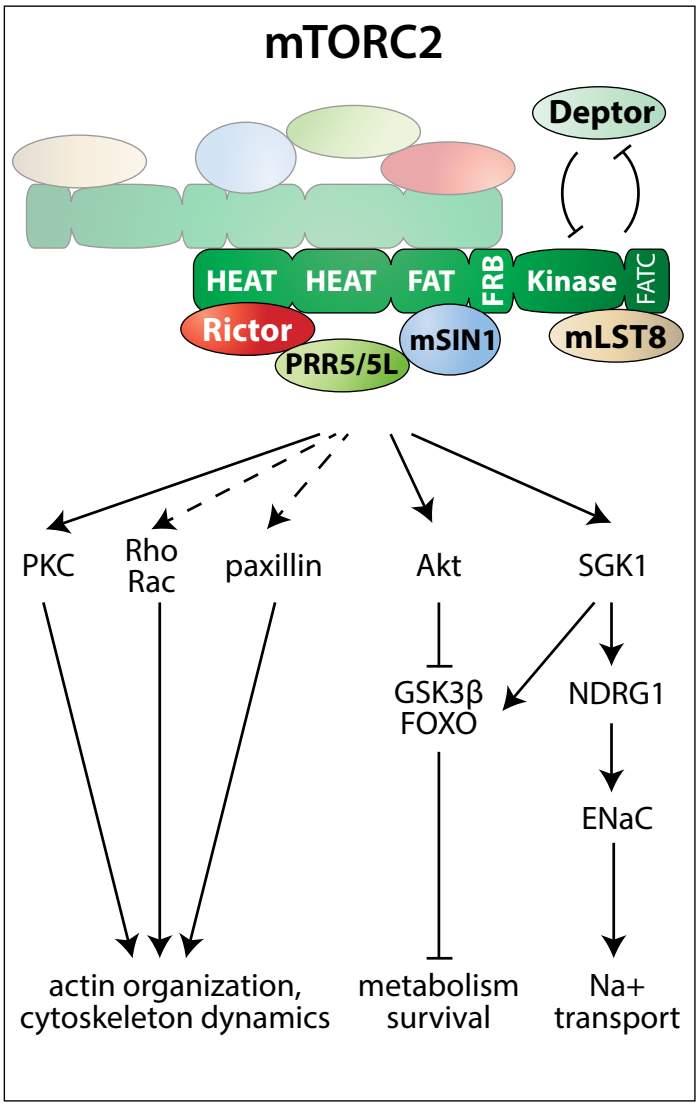

Figure 4: **mTORC2 targets**

1.1.6 *Tissue-specific functions of mTORC2*

The generation of tissue-specific mTOR complex 2 knockout mice helped greatly in elucidating the diverse roles that mTORC2 plays in multicellular organisms.

1.1.6.1 *Adipose Tissue*

Two groups independently reported the generation of adipose tissue specific Rictor knockout mice (Cybulski et al., 2009; Kumar et al., 2010). One remarkable phenotype of these mice is their increase in body and organ size, a phenotype that is even stronger after putting the mice on a high fat diet. This implies the secretion of a unidentified factor from the adipose tissue to other organs such as the liver or the pancreas. Furthermore, the adipose tissue specific Rictor knockout mice are hyperinsulinemic and insulin resistant. Adipogenesis is not affected by mTORC2 knockout.

1.1.6.2 *Muscle*

Rictor knockout in the muscle was reported by two groups (Bentzinger et al., 2008; Kumar et al., 2008). While Kumar *et al.* observed a defect in glucose transport, Bentzinger *et al.* reported no metabolic changes in their mice. Overall, mTORC2 in the muscle seems to play only a secondary role, compared to mTORC1. This may be due to the fact that Akt S473 phosphorylation, for unknown reasons, is not completely abolished upon Rictor knockout in this organ.

1.1.6.3 *Brain*

Knockout of Rictor in neuronal cells leads to the development of schizophrenia-like symptoms due to hypodopaminergia (Siuta et al., 2010). This might explain why certain mental disorders exhibit deregulation of Akt signaling in the brain and why mTORC2 has been linked to neurodegenerative disorders such as Alzheimer's and Parkinson's disease (Beaulieu et al., 2009).

1.1.6.4 *β-cells*

Knockout of Rictor in pancreatic β-cells leads to a mild hyperglycemia and glucose intolerance (Gu et al., 2011). Mice are furthermore hypoinsulinemic because of a reduced beta cell mass.

1.1.6.5 *Liver*

Three independent knockout mice for Rictor in the liver have been described (Hagiwara et al., 2012; Lamming et al., 2012; Yuan et al., 2012). The main phenotype in these mice is reduced glucokinase and SREBP1c activity in the liver, leading to constitutive gluconeogenesis,

impaired glycolysis and lipogenesis. Mice are hyperglycemic, hyperinsulinemic and hypolipidemic.

1.1.6.6 *Immune system*

Rictor knockout in double or single positive T cells show that mTORC2 is crucial for the differentiation into T helper 1 (TH1) and TH2 cells, possibly by regulating STAT signaling (Delgoffe et al., 2009). Partial Sin1 disruption in mice leads to a defective B cell development, probably due to the disruption of Akt2 regulated rag1 and il7r expression (Lazorchak et al., 2010).

1.1.6.7 *Kidney*

Knockout mice for PRR5 were recently described and interestingly show no defect in Akt-S473 phosphorylation. However the authors observed in the kidney a reduced phosphorylation of SGK1 (Pearce et al., 2011). The physiological importance of PRR5/5L remains unclear.

1.1.6.8 *Podocytes*

Podocyte-specific deletion of Rictor leads only to a mild phenotype. Mice exhibit stress-induced BSA overload and increased albuminuria (Gödel et al., 2011).

1.1.7 *mTORC2 function in human disease*

The mTOR network contains several proteins that are directly involved in tumorigenesis. In the mTORC1 signaling network there are the tumor suppressors TSC1 and TSC2, LKB1 and the protooncogene Akt. Loss of the tumor suppressors leads to the development of benign tumors known as hamartomas, whereas Akt amplification has been linked to more aggressive tumors.

Loss of PTEN, while affecting also mTORC1, seems to primarily activate mTORC2 and leads to the development and progression of cancers (Guertin et al., 2009). Indeed, mutations of the PI3K pathway are present in over 50% of all tumors (Yuan and Cantley, 2008). Furthermore, mTORC2 activity in some cancers might also be due to overexpression of Rictor (Masri et al., 2007).

While originally only mTORC1 driven tumors could be targeted in clinical studies because of the mTORC1 specificity of rapamycin and its analogues (rapalogues), a novel line of inhibitors including dual mTOR/PI3K inhibitors and mTOR kinase inhibitors, are capable of targeting mTORC2 driven tumors as well. The rapalogues generally have only a slight effect on patient outcome and this only on a subset of cancer types. This has been attributed to inhibition of the negative feedback loop (O'Reilly et al., 2006; Tabernero et al., 2008). A second

limitation of this line of treatment is the only recently appreciated incomplete inhibitory effect of rapamycin on mTORC1 activated 4E-BP1 (Thoreen et al., 2009; Choo et al., 2008; Feldman et al., 2009), thus potentially not blocking 4E-BP1 driven cancer cell proliferation (Dowling et al., 2010). A growing number of clinical trials using the latest generation of mTOR inhibitors are being rolled out and it remains to be seen whether they will present the anticipated benefit over rapalogues.

Apart from cancer, mTORC2 has been linked to a number of diseases including diabetes, neurodegeneration and possibly aging (Laplante and Sabatini, 2012). These relationships will be explored further in the context of mitochondria and MAM.

1.1.8 *Perspectives in mTORC2 research*

Very little is known about how association with ribosomes contributes to mTOR complex 2 activation. Furthermore, it is unclear how the relatively large amount of ribosomes compared to mTORC2 can activate mTORC2 acutely (in other words, why is mTORC2 not always active?). It should be clarified whether the ribosome-mTORC2 binding directly activates mTORC2 kinase activity, or whether it merely allows localization and targeting of mTOR complex 2 to its substrates. Furthermore, the molecular link between PI3K activity and ribosome-mTORC2 association is uncharacterized.

The consequence of the known post-translational modifications of mTORC2 on its activity are still very poorly understood. Another missing development in the study of mTORC2 signaling are mTORC2 specific inhibitors, that might not only be helpful as research tools but also in clinical applications.

Finally, the localization of mTORC2 is not well understood, especially in the context of its activity. Many early studies hypothesized that mTORC2 activity is mainly restricted to the plasma membrane (Song et al., 2005), possibly since PDK1 is localized to the plasma membrane. As will be explored later, mTORC2 has also been physically or functionally linked to both the endoplasmic reticulum (ER) and mitochondria.

1.2 ENDOPLASMIC RETICULUM (ER)

1.2.1 *Structure and Function*

The ER can be divided into 3 major parts that are nevertheless interconnected: the nuclear envelope, the rough and the smooth ER (RER and SER). It is a highly dynamic structure that needs to be remodeled according to growth conditions. The RER is usually organized in sheets and marked by the presence of attached ribosomes, while the SER is more tubular and convoluted (Voeltz et al., 2002). Most membrane proteins are shared between the SER and the RER. The SER is only abundant in some cell types and its function includes steroid synthesis and detoxification. In the following pages, we will mainly focus on the rough ER.

The ER is an extremely versatile organelle that fulfills many essential functions. The most obvious one is its role in protein synthesis, maturation and quality control. Proteins of the eukaryotic secretory pathway are co-translationally translocated into the ER lumen by membrane attached ribosomes. The targeting of these mRNAs is achieved through an SRP (signal recognition particle) dependent mechanism. SRP binds the emerging peptide from the ribosome upon recognition of the signal sequence, and then pauses translation. The complex is then targeted to the translocon complex on the ER, where translation resumes (Ng et al., 1996). It should be noted however that there are also SRP independent and posttranslational translocation mechanisms into the ER. Yeast cells devoid of SRP are viable, and adapt their growth and secretory pathway to this condition (Siegel, 1995). Interestingly, ER-bound ribosomes can stay bound at the ER after termination of translation and initiate synthesis of both secretory and cytoplasmic proteins (Potter and Nicchitta, 2000).

The ER is furthermore an important platform for signaling. Some enzymatic reactions can occur more efficiently if the dimensionality of the cytoplasm is reduced by localizing the reaction to the surface of a membrane. One example is Ras signaling, which occurs not only at the plasma membrane but also at the ER (Chiu et al., 2002).

The ER is the main calcium store in a cell, and this function will be discussed later on page 21.

1.2.2 *mTORC2 and the ER*

mTORC2 was thought for a long time to be associated only with the plasma membrane, since mTORC2 is activated by PI3K signaling and PIP_3, and since Akt can localize to the plasma membrane through its PH domain. Recently it became clear however, that mTORC2 signaling might not be restricted to the plasma membrane. In rat liver extracts, insulin stimulation translocates phosphatidylinositol 3-kinase

(PI3K) to the ER (Daniele et al., 1999). ER stress inhibits mTORC2 (Chen et al., 2011; Appenzeller-Herzog and Hall, 2012; Hosoi et al., 2007). The observation that PDK1 mediated T308 phosphorylation is affected by ER stress indicates an intact PI3K-mTORC2-Akt signaling at the ER (Yung et al., 2011). A link between mTORC2-Akt signaling and Grp78 or Bip, an ER chaperone upregulated upon ER stress, has been suggested by multiple authors (Lin et al., 2011; Yung et al., 2011; Dai et al., 2010). The exact relationship between mTORC2 signaling and Bip remains unclear. In Chlamydomonas however, TOR controls Bip phosphorylation (Díaz-Troya et al., 2011).

In yeast, TORC2 is peripherally membrane associated and is at or close to the plasma membrane (Wedaman et al., 2003; Sturgill et al., 2008; Kunz et al., 2000), a localization that coincides with cortical ER (Voeltz et al., 2002). It was shown, albeit inconclusively, that mTOR contains ER and Golgi localization sequences which may target mTOR to these organelles (Drenan et al., 2004; Liu and Zheng, 2007). Isolated microsomes (containing ER) harbor mTOR complex 2 activity toward Akt (Hresko and Mueckler, 2005). mTORC2 might interact with a specific subpopulation of ribosomes, such as ER associated ribosomes (Zinzalla et al., 2011). In trypanosoma, TORC2 is localized to both the ER and mitochondria (Barquilla et al., 2008). Finally, a preliminary study suggested that mTORC2 is mainly localized to the ER but failed to show any functional significance of this localization (Boulbés et al., 2011).

1.3 MITOCHONDRIA

1.3.1 *Structure and Function*

Mitochondria possess an outer and an inner membrane. The inner mitochondrial membrane (IMM) is folded into the so-called cristae, thereby increasing the surface area of the IMM. This leads to 2 different compartments inside the mitochondria: the matrix, containing the genome and enzymes from the TCA cycle, among others; and the intermembrane space, harboring the components of the electron transport chain that perfom oxidative phosphorylation. The traditional view of discrete vesicular mitochondria has shifted in the past decades to one of a highly dynamic, reticular network of mitochondria, that undergo fusion and fission and that are highly motile. In specialized cells, mitochondria can agglomerate at sites of high energy demand, such as the Ranvier nodes in neurons (Hollenbeck, 2005).

Mitochondria are central for the energy production from the respiratory chain, which takes place on the surface of the inner membrane. Other important functions include lipid oxidation, oxygen radical production and hormone metabolism (Ferguson, 2002). Their role in calcium homeostasis, while less studied, is equally crucial (Pizzo and Pozzan, 2007). Calcium transfer between the ER stores and mitochondria occurs preferentially at MAM (see Section 1.4 on page 21).

Mitochondria are well known for their function in the induction and execution of apoptosis. Initiator caspases such as caspase 8 (activated for example after binding of the Fas ligand or TNFα, or after intrinsic induction) cleave the ER cargo receptor BAP31 to form p20, which induces Drp1/Dlp1 to activate mitochondrial fission (Simmen et al., 2005; Breckenridge et al., 2003). Mitochondrial fragmentation offers short-term protection against the propagation of apoptotic calcium waves (Szabadkai et al., 2004). Mitochondrial fragmentation can also lead to mitochondrial permeabilization and trigger cell death (Karbowski and Youle, 2003). Mitochondrial permeabilization is mediated by the permeability transition pore (PTP), a large pore in the IMM that, once opened, leads to mitochondrial swelling, outer membrane (OMM) rupture and release of proapoptotic factors such as cytochrome c into the cytosol.

1.3.2 *mTORC2 and mitochondria*

mTOR can associate with mitochondria (Desai et al., 2002) and interact with the mitochondrial/MAM protein VDAC1 (Ramanathan and Schreiber, 2009). mTORC2 phosphorylated Akt (Akt-pS473) can move to mitochondria (Antico Arciuch et al., 2009). Other studies detected Akt localization to mitochondria (Bijur and Jope, 2003; Miyamoto

et al., 2008). Sirt1, which is involved in the regulation of mitochondrial function (Gerhart-Hines et al., 2007), stimulates mTORC2 (Rui-Hong Wang, 2011). Glycerolipids, synthesized by GPAT1 at the mitochondria and possibly the mitochondria associated ER membrane (Ardail et al., 2003), inhibit mTORC2 activity (Zhang et al., 2012). PINK1 (PTEN induced kinase 1), a mitochondrial protein linked to Parkinson's disease affects mTORC2 function as well (Murata et al., 2011). Furthermore, a genome-wide shRNA screen showed an elevated mitochondrial dependence in mTORC2-addicted cells (Colombi et al., 2011). mTORC2 regulates mitochondrial motility (Wang et al., 2010b). In dorsal root ganglion neurons, NGF activates the PI3K pathway to modulate mitochondrial docking and reorganization in an actin dependent way, likely via mTORC2 (Chada and Hollenbeck, 2004).

1.4.1 *Discovery and Structure*

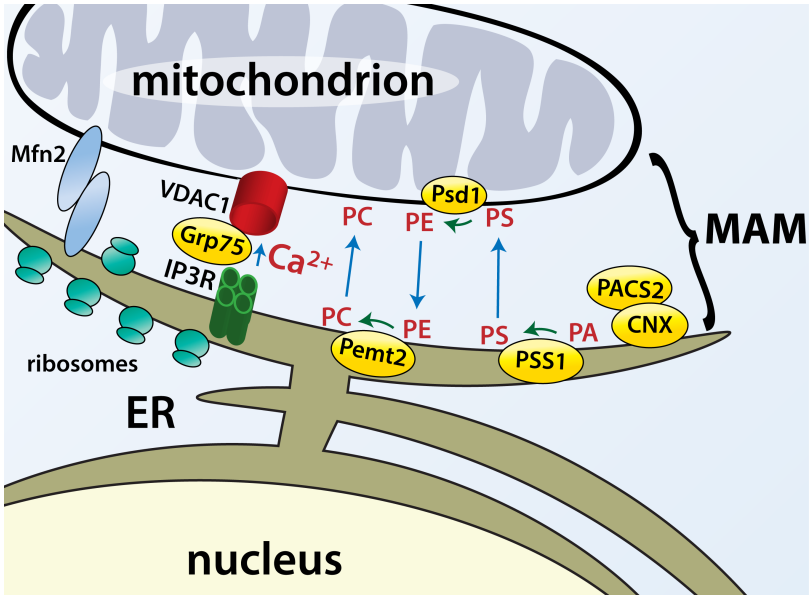

Figure 5: **MAM structure**

Close association of the ER with mitochondria has been observed since the early 1960s (RUBY et al., 1969; Marsh et al., 2001; Mannella, 2006; Mannella et al., 1998; COPELAND and DALTON, 1959; Lewis and Tata, 1973; Morré et al., 1971). This structure, characterized first in mammalian cells, was termed mitochondria associated ER membrane (MAM) (Vance, 1990). It was later also isolated from yeast cells (Gaigg et al., 1995; Zinser et al., 1991). Yeast cells have around 80-110 connections between ER and mitochondria per cell (Achleitner et al., 1999). Approximately 20% of the mitochondrial surface in a cell is surrounded by MAM in mammalian cells (Rizzuto et al., 1998). This led to the hypothesis that mitochondrial-ER communication occurs in a quasi-synaptic structure that is MAM as opposed to vesicular traffic (Simmen et al., 2005).

Initially, it was thought that MAM would form only at the smooth ER (Goetz et al., 2007; Wang et al., 2000), but connections with both SER and RER have been confirmed and the latter type seems to be predominant depending on the cell type (Csordas et al., 2006; Zhang et al., 2011). Distances between the two organelles are roughly 10nm for SER and 25nm for RER. Although the membranes from MAM originate from the ER, its lipid and protein composition differ from that of the ER membranes. While less studied, the mitochondrial membrane at the MAM contact site is also enriched for specific enzymes and lipids. Thus, the term MAM is generally used for the actual contact site between ER and mitochondria (Figure 5, page 21).

PACS2 was identified in 2005 to play a role in the maintenance of MAM (Simmen et al., 2005). PACS2 traffics certain MAM components to and from MAM. For example, it binds unphosphorylated Bid after induction of apoptosis, traffics it to MAM and leads to the release of cytochrome c from mitochondria (Simmen et al., 2005). PACS2 is also important for the translocation of calnexin to MAM (Myhill et al., 2008). Interestingly, PACS2 is regulated by Akt phosphorylation, and this protects cells from apoptosis (Aslan et al., 2009). However it is unknown whether this phosphorylation affects MAM formation.

One of the MAM tethers is the IP3R-Grp75-VDAC1 complex that links the ER via IP3R to mitochondria via VDAC1 (Szabadkai et al., 2006). While both IP3R and VDAC1 are present in the mitochondrial and ER membranes respectively, they are highly enriched at MAM and connected physically via Grp75. IP3R and VDAC1 had previously been linked to MAM and are central components of ER-mitochondrial calcium transfer (Gincel et al., 2001; Rapizzi et al., 2002). VDAC1 also shuttles metabolites such as ATP out of the mitochondria (Vendelin et al., 2004). Several isoforms of the IP3R exist and they all function as ER/MAM calcium channels. While IP3R3 is mostly enriched at MAM and functions as a tightly regulated calcium release channel, the other IP3R isoforms, distributed more equally throughout the ER, might be more important in the basal calcium homeostasis.

A second MAM tether between ER and mitochondria was identified in 2008. Mitofusin 2 (Mfn2) can form homo or hetero dimers with Mfn1 in order to connect the ER and the mitochondria (de Brito and Scorrano, 2008). A third tether called ERMES was discovered in yeast and does not have a mammalian homolog (Kornmann et al., 2009). It should be noted that yeast MAM might have different functions shifted more toward lipid trafficking. In yeast, the ER is not the main calcium store and yeast mitochondria do not possess the calcium uniporter (de Brito and Scorrano, 2010).

Recently, Rab32 was discovered to be a modulator of MAM formation that regulates apoptosis by targeting PKA to MAM and mitochondria (Bui et al., 2010). Palmitoylation can affect targeting of certain proteins such as TMX and calnexin to MAM (Lynes et al., 2011). Information about the proteomic composition of MAM comes from two MS studies of this subdomain. The authors discovered a number of proteins at MAM that require further characterization in this context, e.g. the hexokinase, the TCP1 complex or Bip (Zhang et al., 2011; Poston et al., 2011). An interesting finding from these studies was the presence of the translation machinery at MAM. Palmitoylated calnexin is a key component of the ribosome-translocon complex (Lakkaraju et al., 2012), which together with the fact that palmitoylated calnexin preferentially goes to MAM (Lynes et al., 2011) suggests a functional role of ribosomes at MAM.

The signals that control MAM formation are largely unknown. One study demonstrated that TGFβ exerts a regulatory role toward MAM formation (Pacher et al., 2008). Ionomycin treatment disrupts MAM, probably through the sudden release of the calcium stores into the cytosol (Wang et al., 2000; Goetz et al., 2007). MAM formation is a highly dynamic process (Rizzuto et al., 1998), and a better understanding of the signaling upstream is strikingly still lacking.

A selection of recent, MAM-related reviews

Lynes and Simmen (2011)	ER function in MAM and PAM
Simmen et al. (2010)	Oxidative phosphorylation
Williamson and Colberg-Poley (2009)	Viral reproduction at MAM
Pinton et al. (2008)	Calcium and MAM
Vance and Shiao (1996)	Lipid trafficking at MAM
Grimm (2012)	MAM and apoptosis
Schon and Area-Gomez (2010)	Neurological disease and MAM
Giorgi et al. (2009)	General MAM review
Fujimoto and Hayashi (2011)	General MAM review
Hayashi et al. (2009)	General MAM review
Glancy and Balaban (2012)	Calcium and energetics
Michel and Kornmann (2012)	MAM in yeast
Olson et al. (2012)	Mitochondrial calcium uptake
Raturi and Simmen (2012)	General MAM review
Bravo et al. (2012)	ER stress and MAM
Leem and Koh (2012)	Diabetes and MAM
Bononi et al. (2012)	MAM and calcium
Palmer et al. (2011)	Mitochondrial morphology
Patergnani et al. (2011)	MAM and calcium
Malhotra and Kaufman (2011)	ER stress and MAM
Decuypere et al. (2011)	MAM, apoptosis and autophagy
Osman et al. (2011)	Phospholipid synthesis at MAM
Baltzer et al. (2010)	Mitochondria and nutrients
de Brito and Scorrano (2010)	General MAM review
Cheng et al. (2010)	Mitochondria and insulin
Stiles (2009)	Mitochondria and PI3K signaling
Rizzuto et al. (2009)	MAM and calcium
Lebiedzinska et al. (2009)	ER connections to organelles
Liesa et al. (2009)	Mitochondrial in disease
Vanderheyden et al. (2009)	Regulation of IP3R
Pastorino and Hoek (2008)	Regulation of HK2-VDAC1

1.4.2 *Function of MAM*

MAM has two main functions, namely calcium transfer and lipid synthesis, modification and shuttling. Through these processes, MAM regulates mitochondrial and ER functions, and apoptosis. While both calcium release and lipid synthesis also occur elsewhere at the ER, many key enzymes in lipid biogenesis and ER calcium efflux are clustered around MAM structures, making them hotspots for these processes. Traditionally, the ER is thought to control mitochondria via calcium and lipid transfer. It should be noted though that there is no clear ER-mitochondrial hierarchy, as MAM disruption affects both ER and mitochondrial homeostasis. Furthermore, transfer of calcium and lipids is not unilateral. Certain steps of the lipid synthesis occur on either side of MAM, and even though the ER is the main calcium store, calcium reflux from mitochondria to MAM can modulate the propagation of calcium signaling waves.

Other, less explored functions of MAM include for example the transport of mitochondrial ATP directly to the ER for oxidative protein folding (Koumenis et al., 2002). The proteomic studies discussed above furthermore suggested an important but poorly understood role of MAM in glycolysis (Zhang et al., 2011).

It should be mentioned that another subdomain similar to MAM exists in the ER that associates the ER with the plasma membrane and is termed PAM. This structure is less studied and will not be discussed here (Pichler et al., 2001; Lebiedzinska et al., 2009).

1.4.2.1 *Calcium transfer and apoptosis*

The ER is the major calcium store in the cell. ER calcium storage allows cells and their intrinsic biochemical reactions to become less dependent on plasma membrane mediated ion fluxes. The calcium buffering function of the ER allows calcium to function as a second messenger that is taken up and released under specific conditions (Koch, 1990). As mentioned above, this occurs at MAM upon stimulation of the IP3R calcium channels. IP3R is stimulated by inositol-3-phosphate (IP_3), which in turn is produced at the plasma membrane in response to extracellular stimuli. IP3R function is modulated by many different factors (Hirota et al., 1999). Sigma-1 receptor (Sig-1R) in a complex with Bip interacts with MAM localized IP3R3 to regulate MAM function (Hayashi and Su, 2007). Bcl-2 can interact with IP3R and increase its affinity for IP_3 (White et al., 2005). IP3R has a PACS2 binding site, but this function has not been studied in the context of MAM. ATP can bind IP3R isoforms with different affinities and regulate their activity (Maes et al., 2000). Finally, IP3R can be phosphorylated by active Akt, thereby inhibiting calcium release and apoptosis (Szado et al., 2008; Marchi et al., 2008; Khan et al., 2006; Marchi et al., 2012). Interestingly, Akt was also shown to localize to MAM (Giorgi

et al., 2010), and the Akt-regulated calcium release occurs predominantly at the MAM enriched IP3R3 isoform (Marchi et al., 2012).

Changes in ER calcium can affect oxidative protein folding and hence lead to the induction of the unfolded protein response. While the concentration of cytoplasmic calcium is generally low (10-100nM), rapid increases can be observed under particular conditions, rising up to 2μM due to influx either from the plasma membrane or through release at MAM. Further exit routes for calcium from the ER are the ryanodine receptors, which are primarily important in muscle tissue, and a minor leakage through the translocon pore. Calcium from the cytosol is reimported into the ER by the Sarco-Endoplasmic Reticulum Ca^{2+}-ATPase (SERCA).

Besides the role of the ER in maintaining cytoplasmic calcium homeostasis under normal growing conditions, stimulated calcium release occurs mostly at MAM in order to regulate mitochondrial function (Rizzuto et al., 1998). The cytoplasm is a very poor calcium conductor (less than $10\mu m^2/s$) due to its abundance of calcium buffering proteins. MAM calcium transfer is reminiscent of neuronal signal transduction and is described as being "quasi-synaptic" (Csordas and Thomas, 1999). Similar to neuronal transmission, the spatial and temporal patterns of calcium fluxes seem to encode specific cellular responses (Putney, 1998), and cells can decode not only the amplitude but also the frequency of such oscillations (Dolmetsch et al., 1998; Li et al., 1998).

MAM-released calcium flows from the ER side into the intermembrane space of the mitochondria. Calcium channels through the outer mitochondrial membrane (OMM) via VDAC1. It can then enter the matrix of the mitochondria, however the identity of the IMM calcium uniporter is still poorly defined (De Stefani et al., 2011b). The influx of the positively charged calcium ions necessitates the conservation of the inner membrane potential that would otherwise collapse. VDAC1 is a voltage gated channel that transports ions, nucleotides and calcium. It can change its conformation depending on different conditions, thereby modulating its selectivity and permeability (Giorgi et al., 2009). Under zero and low (negative or positive) transmembrane potentials, VDAC1 has a highly conductive open state with preference for anionic compounds such as ATP (Shoshan-Barmatz, 2005). Upon VDAC1 closure, the channel exhibits increased permeability for cations and this can lead to an increased mitochondrial influx of calcium. Permeability toward ATP however is completely abolished upon closure of VDAC1. VDAC1 closure can be regulated by different proteins. Association of VDAC1 with hexokinase 1 or 2 (HK1/HK2) promotes cell growth by directly coupling mitochondrial ATP export to glycolysis (Bryson et al., 2002; Pastorino et al., 2002). VDAC1-HK2 association is enhanced in certain tumor cells (Wolf et al., 2011). Detachment of HK2 from VDAC1 induces VDAC1 clo-

sure, resulting in an increased mitochondrial calcium uptake, an enhanced respiration rate (since calcium stimulates the TCA cylce) and an increased mitochondrial membrane potential. (Stiles, 2009; Chiara et al., 2008). Interestingly, HK2-VDAC1 binding is stimulated by Akt mediated phosphorylation of HK2 (Gottlob et al., 2001; Bryson et al., 2002; Chiara et al., 2008; Miyamoto et al., 2008) and by GSK3β mediated phosphorylation of VDAC1 (Pastorino et al., 2005). Even after termination of the initial MAM calcium pulse, OMM and intermembrane enzymes inside mitochondria can "remember" this pulse and change their respective activities (Lasorsa et al., 2003; Hansford and Zorov, 1998), an effect that was termed "metabolic memory" (Jouaville et al., 1999). Calcium stimulates the TCA cycle and ATP synthesis directly via the pyruvate-, α-ketoglutarate- and isocitrate dehydrogenases (Berridge, 2002).

Calcium exits the mitochondria either through VDAC1, through NCLX or through a Ca^{2+}/Na^{+} exchanger (Saris and Carafoli, 2005), and efflux of mitochondrial calcium at MAM might play a role in the propagation of calcium waves (Boitier et al., 1999; Landolfi et al., 1998).

Disruption of MAM by depletion of proteins such as PACS2, Sig-1R or Mfn2 leads to an increase of cytosolic calcium upon IP3R stimulation, as the calcium now leaks into the cytoplasm instead of entering mitochondria (Simmen et al., 2005; de Brito and Scorrano, 2008; Hayashi and Su, 2007). Unexpectedly however, Mfn2 disruption also led to an increase in mitochondrial calcium uptake as these cells possess larger ER calcium stores and, accordingly, release more calcium (de Brito and Scorrano, 2008). This increased calcium release might be a compensation to cover for the decreased MAM calcium transfer.

By modulating calcium homeostasis, MAM regulates ER chaperone assisted folding of proteins and is thus implicated in ER stress regulation. However MAM released calcium also plays a crucial role in the induction of apoptosis (Berridge and Bootman, 1998). As mentioned earlier, MAM-calcium fluxes can trigger different responses depending on the properties of the calcium flux. Enhanced mitochondrial calcium influx at MAM is a priming factor that lowers the apoptotic threshold until a second insult hits the cell and triggers the onset of apoptosis (Pinton et al., 2001; Szalai et al., 1999). MAM structures should not be pigeonholed as being either pro- or anti-apoptotic. It is more accurate to see MAM as a gatekeeper that can mediate or prevent apoptosis dynamically under specific conditions. Accordingly, disruption of MAM components can both induce or protect from apoptosis (Giorgi et al., 2012).

MAM mediated calcium influx into mitochondria is important in the brown adipose tissue in order to maintain and regulate thermogenesis (Kuba et al., 2007; de Meis et al., 2010).

1.4.2.2 *Lipids*

MAM has an exceptionally high capacity for lipid synthesis (Rusiñol et al., 1994). MAM is now recognized as being a site of phospholipid synthesis and transfer (Voelker, 2000; Vance, 1990, 2004). The best characterized example is phosphatidylethanolamine (PE) biosynthesis. Phosphatidylserine (PS) is synthesized on the ER side of MAM by phosphatidylserine synthase and is then transported to the mitochondrial side where it undergoes decarboxylation to form PE (Ardail et al., 1989; Fagone, 2009) (Figure 5, page 21). Later work also uncovered the generation of sphingolipids and ceramides at MAM (Ardail et al., 2003; Bionda et al., 2004). Some of these enzymes are highly enriched at MAM and serve as MAM markers (ACSL4, PSS1 and PEMT2) (Piccini et al., 1998; Stone and Vance, 2000; Vance et al., 1997). The role of these lipids are diverse and include regulation of mitochondrial and ER morphology, and apoptosis (Stiban et al., 2008).

1.4.2.3 *MAM function in disease*

ACSL4 (Long-Chain Acyl-CoA Synthetase 4, FACL4) is an enzyme that is highly enriched at MAM and has been linked to Alport syndrome, elliptocytosis and mental retardation (Piccini et al., 1998). The molecular basis for the human neurodegenerative disease NCL (neuronal ceroid lipofuscinosis) is caused by a reduction of lipid trafficking due to MAM disruption (Vance, 1997). Another lipid trafficking disease is GM1-gangliosidosis, a neurodegenerative disease where GM1 accumulates at MAM (Sano et al., 2009). Loss of Mfn2 leads to Charcot-Marie-Tooth neuropathy type 2a (Feely et al., 2011). Sig-1R is a target for many neurosteroids and neuroleptics (Decuypere et al., 2011). Presenilins, loss of which causes familiar Alzheimer's disease (AD), localize to MAM. Thus, AD could be a disorder of the MAM (Schon and Area-Gomez, 2010; Zampese et al., 2011). Interestingly, mTORC2 signaling is also implicated in AD (Pei and Hugon, 2008) and other parallels between these neurological syndromes and the phenotypes of defective mTOR signaling exist (Beaulieu et al., 2009; Siuta et al., 2010; Dummler and Hemmings, 2007; Meikle et al., 2008). It is unclear why many MAM-linked disease states have neurological phenotypes. Likely, MAM function is particularly important in neurons (Celsi et al., 2009), as neuronal activity consumes a large amount of mitochondrially produced ATP (Kann and Kovács, 2007).

MAM plays an important but poorly defined role in viral infection and replication. Several viral proteins from CMV and HCV localize to MAM (Bozidis et al., 2008; Sheikh et al., 2008; Zhang et al., 2011; Horner et al., 2011). Interestingly, CMV infection, which activates mTORC2 (Clippinger et al., 2011) also increases the amount of ribosomes at the MAM (Zhang et al., 2011). The HIV protein Nef in-

teracts with PACS2 (Atkins et al., 2008). Finally, MAVS initiates the innate immune response at MAM (Horner et al., 2011).

As MAM is involved in the regulation of energy metabolism and cell death, it is interesting to see that mitostatin, a MAM regulator, is a tumor suppressor deleted in a variety of tumors (de Brito and Scorrano, 2010; Vecchione et al., 2009). Grp75 is overexpressed in various tumors and controls cell proliferation (Kaul et al., 2007). PACS2 is mutated in up to 40% of sporadic colorectal cancer biopsies (Anderson et al., 2001). Recently, the tumor suppressor PML was found to localize to MAM, where it forms a complex with Akt and IP3R, modulating Akt activity via PP2a (Giorgi et al., 2010). Finally, MAM plays a role in the pathogenesis of type 2 diabetes (Leem and Koh, 2012), possibly through its influence on glycolysis (Zhang et al., 2011).

Timeline of important MAM-related publications

Verfaillie et al. (2012)	PERK at MAM mediates ER stress
Sebastián et al. (2012)	Liver-specific deletion of Mfn2
Fujimoto et al. (2012)	Cholesterol regulates MAM
Marchi et al. (2012)	Akt regulates calcium release via IP3R3
Poston et al. (2011)	MAM proteome
Zhang et al. (2011)	MAM proteome
Boulbés et al. (2011)	mTORC2 localization to the ER
Horner et al. (2011)	HCV replicates at MAM
De Stefani et al. (2011a)	VDAC1 relays apoptotic signals
Colombi et al. (2011)	Mitochondrial and mTORC2 addicted cells
Murata et al. (2011)	PINK1 activates mTORC2
Zinzalla et al. (2011)	mTORC2 binds to ribosomes
Zampese et al. (2011)	Presenilin is at MAM
Lynes et al. (2011)	Palmitoylation signals MAM localization
Giorgi et al. (2010)	PML and Akt are at MAM
Bui et al. (2010)	Rab32 modulates MAM
de Meis et al. (2010)	Thermogenesis by MAM in BAT
Behrends et al. (2010)	mLST8 interacts with Grp75
Ramanathan and Schreiber (2009)	mTOR is associated to VDAC1
Kornmann et al. (2009)	Identification of the ERMES MAM tether
Aslan et al. (2009)	Akt phosphorylates PACS2
Stone et al. (2009)	DGAT2 and triglyceride synthesis at MAM
Antico Arciuch et al. (2009)	Akt-pS473 goes to mitochondria
Sano et al. (2009)	Gangliosides and ER stress at MAM
de Brito and Scorrano (2008)	Mfn2 is MAM tether
Pacher et al. (2008)	TGF-β regulation of MAM

Marchi et al. (2008)	Akt phosphorylates IP3R
Barquilla et al. (2008)	TORC2 at ER and mitochondria
Myhill et al. (2008)	PACS2 regulates calnexin distribution
Miyamoto et al. (2008)	Akt phosphorylates HK2
Stiban et al. (2008)	Ceramides are important for MAM
Bozidis et al. (2008)	CMV replicates at MAM
Szado et al. (2008)	Akt phosphorylates IP3R
Diaz-Troya et al. (2008)	TOR is at ER in Chlamydomonas
Goetz et al. (2007)	MAM is regulated by calcium
Hayashi (2007)	Sig-1R is at MAM
Szabadkai et al. (2006)	IP3R-Grp75-VDAC1 is a MAM tether
Khan et al. (2006)	Akt phosphorylates IP3R
Simmen et al. (2005)	PACS2 controls MAM
Bionda et al. (2004)	Ceramide metabolism at MAM
Ardail et al. (2003)	Sphingolipid biosynthesis at MAM
Stone and Vance (2000)	PS synthesis at MAM
Szalai et al. (1999)	IP3 regulates apoptosis via calcium
Achleitner et al. (1999)	MAM quantification in yeast
Csordas and Thomas (1999)	MAM is a calcium "synapse"
Landolfi et al. (1998)	MAM transfers calcium
Rizzuto et al. (1998)	MAM transfers calcium
Mannella et al. (1998)	EM tomography of MAM
Vance (1997)	MAM involved in lipofuscinosis
Gaigg et al. (1995)	Isolation of MAM from yeast
Shiao and Lupo (1995)	MAM and phospholipid synthesis
Rusiñol et al. (1994)	Lipid synthesis at MAM
Vance (1990)	Lipid synthesis at MAM
Morré et al. (1971)	EM evidence of MAM
RUBY et al. (1969)	EM evidence of MAM
COPELAND and DALTON (1959)	EM evidence of MAM

1.4.3 *mTORC2 and MAM*

As mentioned earlier, several independent lines of evidence suggest a functional link between the ER, mitochondria and mTORC2 signaling. MAM, a physical link between ER and mitochondria, presents further observations that might link it to mTORC2. First, in yeast, TORC2 regulates ceramides and sphingolipid metabolism (Aronova et al., 2008) and this regulation might be conserved in mammalian cells (Powers

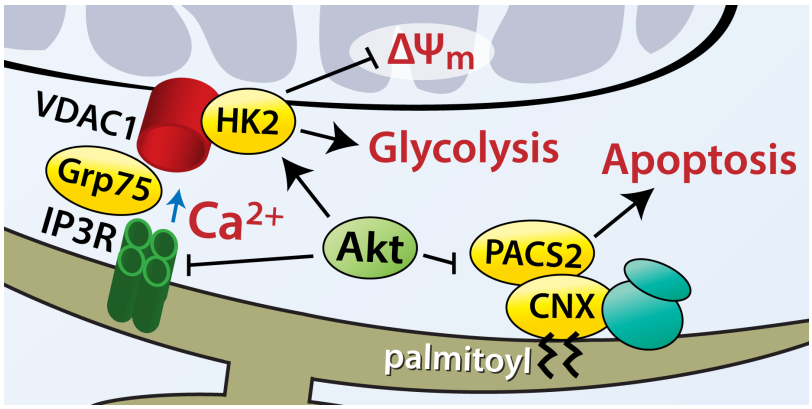

Figure 6: **Akt signaling at MAM**

and Aronova, 2010; Aronova et al., 2008). MAM, on the other hand, is one of the hotspots of ceramide synthesis and sphingolipid transport (Ardail et al., 2003; Bionda et al., 2004; Rusiñol et al., 1994). Second, mTORC2 was isolated from lipid rafts (Partovian et al., 2008). MAM is also a lipid raft (Poston et al., 2011) and might be the source for mTORC2 in the lipid raft fractions. Third, the TCP-1 complex, found at MAM, genetically interacts with TOR2 in yeast (Schmidt et al., 1996; Poston et al., 2011). Forth, TGFβ inhibits both mTORC2 activity and MAM function. Fifth, PEMT2, a MAM resident protein, downregulates PI3K - Akt signaling upon overexpression (Zou et al., 2002). Sixth, Akt localizes to MAM (Giorgi et al., 2010). Seventh, Akt-pS473 phosphorylation is stimulated by phosphatidylserine, a phospholipid that is produced and enriched at MAM (Stone and Vance, 2000). Finally, the phenotypes of deleting mTORC2 and Mfn2 in the liver of mice are strikingly similar and include glucose intolerance, hyperinsulinemia and enhanced gluconeogenesis (Hagiwara et al., 2012; Sebastián et al., 2012).

mTOR complex 1 localization to lysosomes is crucial for its activation. mTOR complex 2 localization has been linked to multiple organelles and is generally less studied. This lack of understanding of basic mTORC2 signaling is even more striking when considering that mTORC2 deregulation is implicated in a wide spectrum of human diseases including cancer and diabetes. As discussed above, mTORC2 has been linked to both the ER and mitochondria, both functionally and physically. MAM is a central hub in cells where ER and mitochondrial functions meet and are synchronized by signaling. Importantly, MAM formation is not well understood, especially considering the upstream signaling. For example, insulin regulates ER and mitochondrial metabolism, however it is unclear whether it also affects MAM. Based on the cumulative evidence presented above, I asked whether mTORC2 could localize to MAM and whether mTORC2 could mediate growth factor signals to MAM.

2 RESULTS

2.1 MTORC2-AKT SIGNALING IS PHYSICALLY AND FUNCTIONALLY AT MAM

The following manuscript forms the basis of my PhD thesis. The introduction of the manuscript overlaps partially with the general introduction at the beginning of the thesis (page 1). The cumulative bibliography from the manuscript and the thesis is given on page 99. Manuscript submitted to *Molecular Cell*.

mTORC2-AKT SIGNALING IS PHYSICALLY AND FUNCTIONALLY AT MAM

Charles Betz⋆, Daniele Stracka⋆, Marco Colombi⋆, Cristina Baschong⋆, Maud Frieden⋆⋆, Nicolas Demaurex⋆⋆ and Michael N. Hall⋆

⋆ Biozentrum, University of Basel
CH-4056 Basel, Switzerland

⋆⋆ CMU, Rue Michel-Servet 1
CH-1211 Geneva, Switzerland

Corresponding author: Michael N. Hall (m.hall@unibas.ch)

2.1.1 *Abstract*

The target of rapamycin (TOR) is a conserved protein kinase and a central controller of growth. Mammalian TOR complex 2 (mTORC2) regulates AGC kinase family members and is implicated in various disorders including cancer and diabetes. Here we report that mTORC2 is localized to the endoplasmic reticulum (ER) and the ER subcompartment termed mitochondria associated ER membrane (MAM). mTORC2 localization to MAM was growth factor stimulated, and mTORC2 at MAM interacted with the IP3R-Grp75-VDAC1 ER-mitochondrial tethering complex. mTORC2 deficiency disrupted MAM, causing mitochondrial defects including increased respiration, ATP production, and calcium uptake. mTORC2 controlled MAM integrity and mitochondrial function via Akt mediated phosphorylation of MAM associated proteins IP3R, hexokinase 2 and PACS2. Thus, mTORC2 is at the core of a MAM signaling hub that controls growth and metabolism. These findings may offer new therapeutic strategies against mTORC2 driven disease.

2.1.2 *Introduction*

Mitochondria-associated ER membrane (MAM) is a sub-compartment of the endoplasmic reticulum that forms a quasi-synaptic structure with the mitochondria. Its main function is to facilitate the transfer of lipids and calcium between the two organelles. MAM thereby controls mitochondrial physiology and apoptosis (Csordas and Thomas, 1999; Rizzuto et al., 1998; Duchen et al., 2008). MAM also mediates ER homeostasis and lipid biosynthesis by harboring chaperones and several key lipid synthesis enzymes (Osman et al., 2011; Stone et al., 2009; Ardail et al., 2003; Bionda et al., 2004; Vance, 1990). In mammalian MAM, the ER and mitochondria are physically tethered to each other by the IP3R-Grp75-VDAC1 trimeric complex (Szabadkai et al., 2006) and dimers of the mitofusin proteins Mfn1 and Mfn2 (de Brito and Scorrano, 2008) (Figure S1A). MAM formation is regulated by multiple signaling inputs including calcium and possibly growth factors (Bravo et al., 2012; Bui et al., 2010; Pizzo and Pozzan, 2007; Pawlikowska et al., 2007; Verfaillie et al., 2012; Sebastián et al., 2012). However, the mechanism(s) that controls MAM formation is largely unknown other than it involves recruitment of MAM components by the MAM resident protein PACS2 (Simmen et al., 2005; Myhill et al., 2008). Akt, an AGC family kinase that is also found at MAM (Giorgi et al., 2010), phosphorylates PACS2 (Aslan et al., 2009), but it remains to be determined whether Akt is involved in mediating MAM integrity.

Akt, often upregulated in cancer, also phosphorylates hexokinase 2 (HK2) to promote association of HK2 with the MAM protein VDAC1

(Miyamoto et al., 2008; Gottlob et al., 2001; Pastorino and Hoek, 2008). This association enables HK2, using ATP exiting mitochondria through VDAC1, to phosphorylate glucose and thereby stimulate glycolysis (Stiles, 2009). Conversely, upon inhibition of Akt, HK2 dissociates from VDAC1 causing VDAC1 closure and increased mitochondrial membrane potential (Gottlob et al., 2001). This regulation of HK2 by Akt has been proposed to account for enhanced glycolysis in cancer cells, also known as the Warburg effect (Wolf et al., 2011). Furthermore, Akt regulates calcium release from MAM by phosphorylating the IP_3 receptor (IP3R), thereby controlling apoptosis (Szado et al., 2008; Marchi et al., 2008, 2012; Khan et al., 2006). Thus, MAM is increasingly recognized as a signaling hub controlling cell physiology, and is implicated in a wide spectrum of diseases including cancer, neurodegenerative disorders, inflammation, and infection (Raturi and Simmen, 2012).

The Target of Rapamycin (TOR) pathway is a cellular signaling cascade that, like mitochondria, is present in all eukaryotes (Soulard et al., 2009; Laplante and Sabatini, 2009a). It integrates and relays signals from both extra- and intra-cellular sources (e.g., growth factors, nutrients and cellular energy levels), and thereby instructs the cell to grow. TOR is found in two structurally and functionally distinct protein complexes that in mammalian cells are termed mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) (Wullschleger et al., 2006). mTORC2 comprises mTOR, Rictor, mLST8, Sin1 and protor (also known as PRR5) (Cybulski and Hall, 2009), and phosphorylates AGC kinases such as Akt, SGK1 and PKC, all of which are linked to cancer and diabetes (Oh and Jacinto, 2011; Pearce et al., 2010). Growth factors activate mTORC2 by promoting mTORC2-ribosome association in a PI3K dependent manner (Oh et al., 2010; Zinzalla et al., 2011). mTORC2 is anti-apoptotic, presumably via its role in phosphorylating and activating Akt and thereby inhibiting pro-apoptotic Bad and FoxO3 (Thedieck et al., 2007; Zinzalla et al., 2011; Sarbassov et al., 2005; Yang et al., 2006).

Recent findings suggest that mTORC2 is at the endoplasmic reticulum (ER), possibly through interaction with ER-bound ribosomes (Boulbés et al., 2011; Zinzalla et al., 2011). mTORC2 phosphorylates Akt at the ER (Hresko and Mueckler, 2005; Boulbés et al., 2011), and mTOR has been proposed to contain an ER-localization domain (Liu and Zheng, 2007) and to sense ER stress (Chen et al., 2011; Appenzeller-Herzog and Hall, 2012). In yeast, TORC2 is peripherally membrane associated and is at or close to the plasma membrane (Wedaman et al., 2003; Sturgill et al., 2008; Kunz et al., 2000), a localization that coincides with cortical ER (Voeltz et al., 2002). TORC2 has also been linked to mitochondria. Barquilla et al. reported that TORC2 in trypanosomes is localized to both ER and mitochondria (Barquilla et al., 2008). mTORC2 regulates the cellular distribution of mitochon-

dria (Wang et al., 2010b), and mTORC2 activated Akt is associated with mitochondria (Antico Arciuch et al., 2009; Miyamoto et al., 2008; Bijur and Jope, 2003). PINK1, a regulator of mitochondrial function, can activate mTORC2 (Murata et al., 2011). Finally, mTORC2-addicted cancer cells exhibit enhanced dependence on mitochondria and HK2 (Colombi et al., 2011). Thus, TORC2 has been physically and functionally linked to both the ER and mitochondria.

Here we investigated the localization of mTOR complex 2. We show that ribosome-bound mTORC2 is at the ER, in particular at MAM. Localization to MAM is growth factor dependent. MAM-associated mTORC2 activates Akt and thereby controls MAM integrity, mitochondrial metabolism, and cell survival. Thus, our findings describe a critical role for mTORC2 in a MAM signaling hub.

2.1.3 *Results*

mTORC2 localizes to mitochondria associated ER-membranes

It has been suggested that mTORC2 is at the ER (Liu and Zheng, 2007; Zinzalla et al., 2011; Boulbés et al., 2011). To examine further mTORC2 localization, we isolated ER from mouse liver and HeLa cell extracts by isopycnic flotation (Stephens et al., 2008) and assayed for mTORC2 by immunoblotting (Figure 8C, page 41). Liver was used because it is a rich source of ER and because previous studies on mTORC2 localization were performed only with cultured cells. 30-60% of mTORC2 (Sin1) was ER associated for liver and HeLa cells, while only $\pm$ 2% of mTORC1 (Raptor) was detected in the ER fraction for liver cells (Figure 7A, page 40 and Figure 8B, page 41). Previous studies have suggested that mTORC2 phosphorylates Akt at the ER (Hresko and Mueckler, 2005; Boulbés et al., 2011). In agreement, we detected T308- and S473-phosphorylated Akt enriched in the ER fractions (Figure 7A, page 40 and Figure 8B, page 41). Furthermore, we confirmed that mTORC2 at the ER is intact and associated with ribosomes (Figure 7B, page 40). Thus, mTORC2 appears to be localized to the ER where it phosphorylates Akt.

To characterize further the subcellular localization of mTORC2, we analyzed Rictor localization by immunofluorescence. Interestingly, the mTORC2 signal overlapped with mitochondria adjacent to ER (Figure 7C, page 40 and Figure 8E, page 41), a staining pattern similar to that of mitochondria associated ER membrane (MAM) proteins (Bui et al., 2010; Lynes et al., 2011; Horner et al., 2011). We next examined whether mTORC2 is MAM associated. First, as visualized by electron microscopy, we detected Rictor at the ER in close proximity to mitochondria (Figure 7D, page 40 and Figure 8D, page 41). Second, as assayed by subcellular fractionation, Rictor was detected in a crude mitochondria fraction but not in a fraction of pure mitochondria that have been stripped of peripheral MAM (Figure 7E, page 40). Third, we detected approximately 15% of total Sin1 and Rictor in a purified MAM fraction (Figure 7F, page 40). Finally, we observed that Rictor and Sin1 co-immunoprecipitated with the ER-mitochondria tethering comples IP3R-Grp75-VDAC1 (Figure 9B, page 44 and Figure 10B, page 45). Grp75 was previously shown in a proteomic study to interact with the mTORC component mLST8 (Behrends et al., 2010) and mTOR was shown to interact with VDAC1 (Ramanathan and Schreiber, 2009). Thus, mTORC2 localizes to ER and to the ER subcompartment MAM where it interacts with an ER-mitochondria tethering complex.

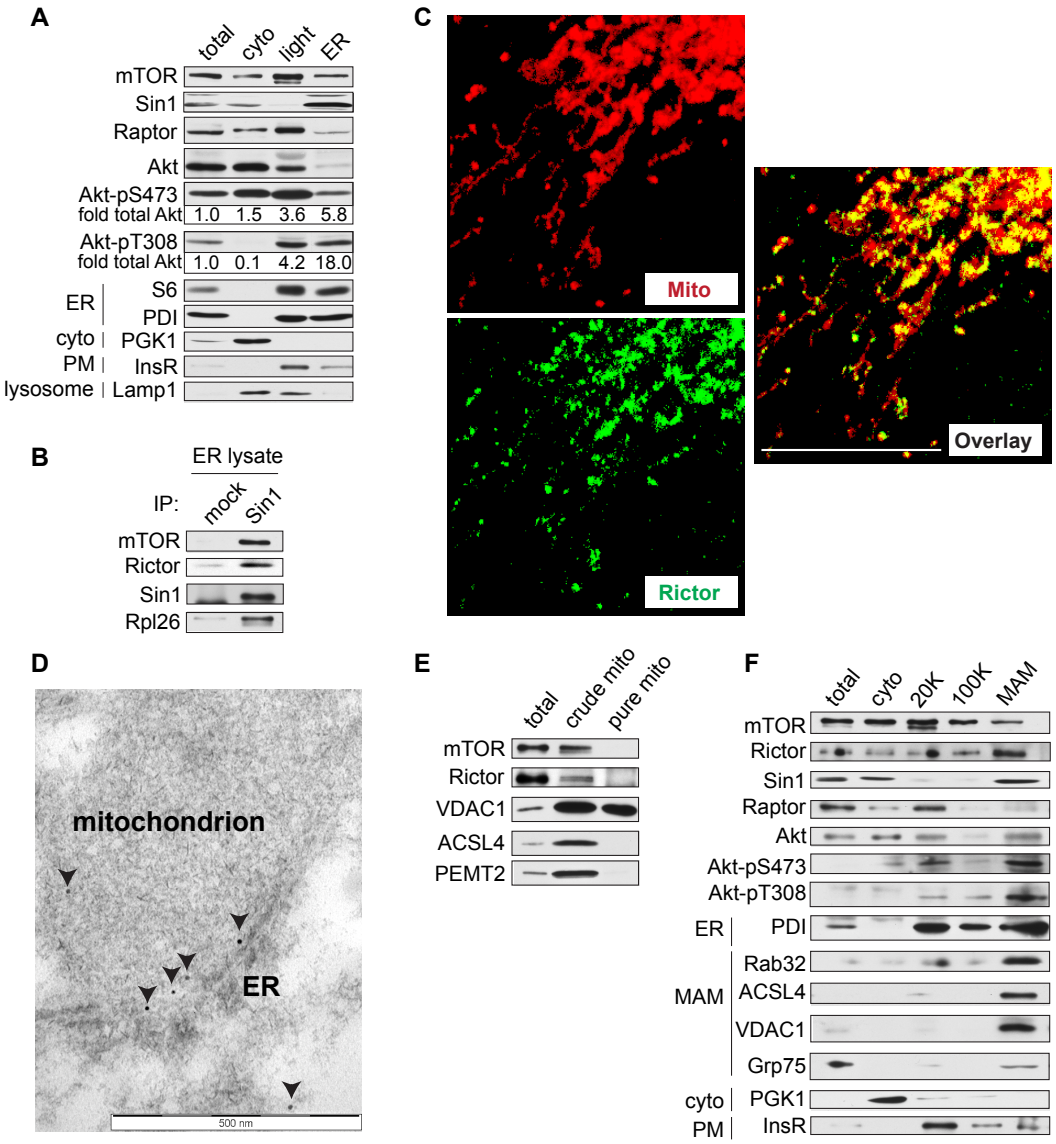

Figure 7: **mTORC2 localizes to MAM**. **(A)** mTORC2 components and Akt are present in the ER fraction in mouse liver cells. Fold total Akt = Ratio of phosphorylated Akt/total Akt levels of respective fraction divided by the ratio of phosphorylated Akt/total Akt of the total fraction. Total = whole cell lysate, cyto = cytoplasmic extract, light = light membrane fraction, ER = heavy membrane fraction from isopycnic flotation. Lysates were pooled from 3 different mouse livers. **(B)** Sin1 co-immunoprecipitates mTOR, Rictor and Rpl26 in mouse liver ER extracts indicating intact and ribosome associated mTORC2. **(C)** Endogenous Rictor localization in U2OS cells overlaps partially mitochondrial marker (mito-RFP). Excerpt from Figure 8C, page 41. **(D)** Rictor (arrows) is localized at the rough ER in close proximity to mitochondria in mouse liver. Bar = 500nm. **(E)** mTOR and Rictor can be detected in crude mitochondrial extracts from mouse liver cells but not in purified mitochondrial extracts. Lysates are pooled from 3 different mouse livers. **(F)** mTORC2 components but not mTORC1 component Raptor are present in purified MAM fraction. Lysates are pooled from 3 different mouse livers. 20K and 100K indicate fractions as described in (Wieckowski et al., 2009).

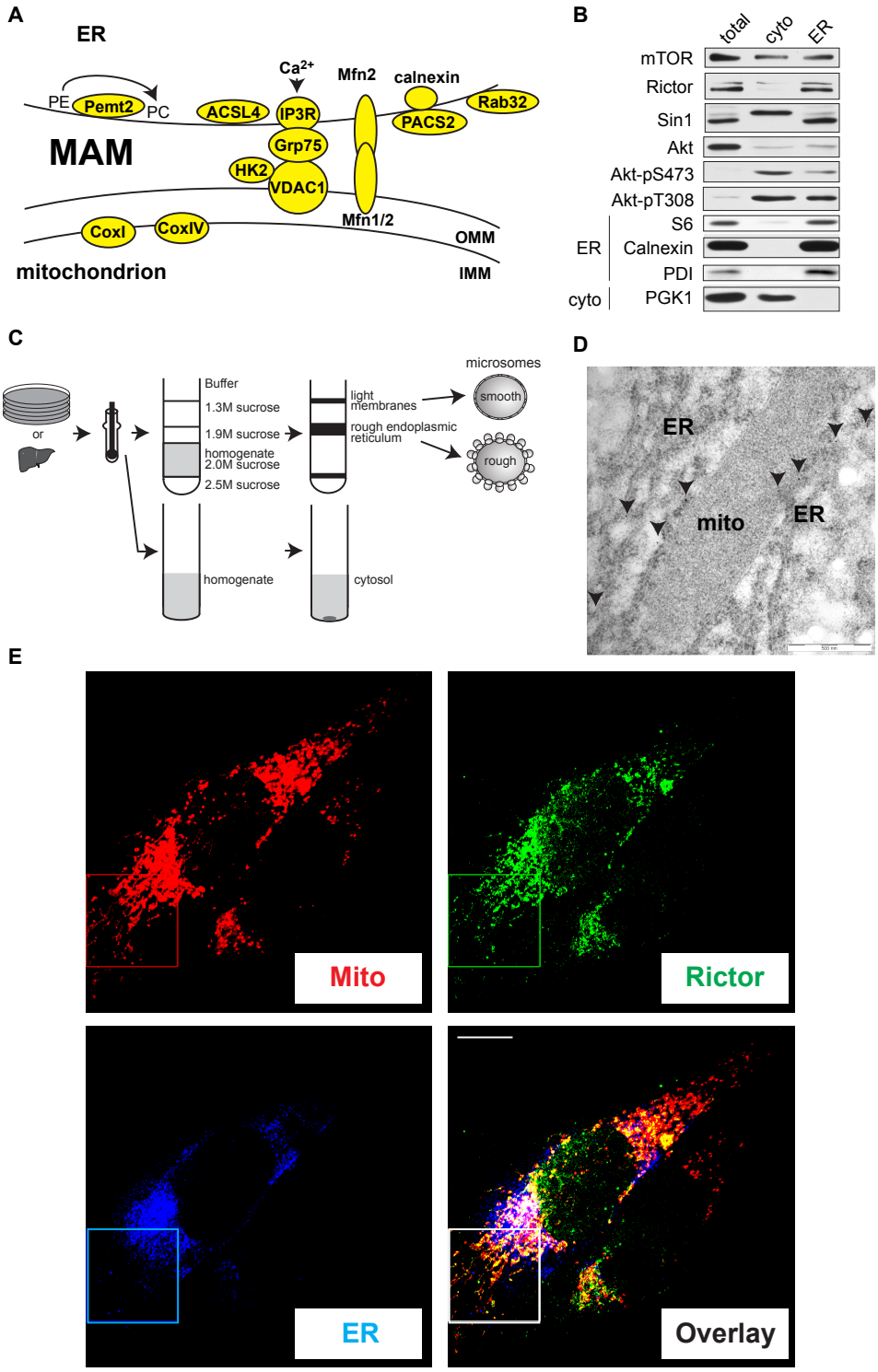

Figure 8: **Supplementary: mTORC2 localizes to MAM. (A)** Schematic overview of the MAM including marker proteins used in this study. **(B)** Isopycnic isolation of ER from HeLa cells. **(C)** Schematic representation for the isopycnic ER isolation protocol, adapted from (Lerner et al., 2003; Stephens et al., 2008). **(D)** Electron micrograph from mouse liver cells staining for Rictor (arrows). **(E)** Rictor localization in U2OS cells overlaps partially with the ER in close proximity of mitochondria. Cells express ER-GFP, mitochondria-RFP and endogenous Rictor.

Localization of mTORC2 to MAM is stimulated by growth factors

Since mTOR complex 2 activity is insulin-PI3K stimulated, we examined whether localization of mTORC2 to the ER and in particular to MAM are regulated by growth factors. We observed 4- to at least 7-fold higher levels of mTORC2 at the ER (Figure 10A, page 45) and at MAM (Figure 9A, page 44) in liver extracts of mice stimulated with insulin. We also detected enhanced interaction between mTORC2 and the MAM complex IP3R-Grp75-VDAC1 after insulin stimulation (Figure 9B, page 44 and Figure 10B, page 45), as assayed by co-immunoprecipitation. Furthermore, we examined by immunofluorescence the localization of Rictor and the ER marker Hsp47 (Figure 9C, page 44), and observed a significant increase in co-localization of Rictor and Hsp47 in cells grown in the presence of serum as compared to serum starved cells. Thus, localization of mTORC2 to the ER and MAM is stimulated by growth factors.

Since activation of mTORC2 involves binding to the ribosome and coincides with ER/MAM localization, we examined whether ER/-MAM localization of mTORC2 requires ribosome binding. Stripping ribosomes from isolated ER (Figure 10C, page 45) and crude mitochondria (Figure 9D, page 44) (consisting of mitochondria and MAM) reduced the binding of mTORC2 to these organelles. Furthermore, we observed an mTORC2 dependent increase in ribosomes at MAM upon growth factor stimulation, suggesting that ribosomes may move to MAM upon mTORC2 binding (Figure 9E, page 44). The above observations together suggest that mTORC2 localization to MAM is dependent on growth factor signaling and interaction with the ribosome.

mTORC2 maintains MAM integrity

We next examined whether mTORC2 regulates MAM integrity. First, we investigated MAM integrity by assaying MAM components in a crude mitochondrial fraction (de Brito and Scorrano, 2008). The amounts of MAM proteins IP3R and Grp75 were reduced in a crude mitochondrial fraction from Rictor knockout cells compared to wild type cells, although total cellular levels of these proteins were unchanged (Figure 11A-B, page 46; Figure 12A and G, page 47). As a second method to assay ER-mitochondria interaction, we monitored the IP3R-Grp75-VDAC1 complex (Szabadkai et al., 2006; de Brito and Scorrano, 2008). Less Grp75 and VDAC1 co-immunoprecipitated with IP3R in Rictor knockout cells (Figure 11C, page 46). Third, we quantified ER-mitochondrial proximity by analysis of 3D confocal images of cells co-expressing fluorescent probes for the ER and mitochondria (Lynes et al., 2011; Bui et al., 2010; Simmen et al., 2005; de Brito and Scorrano, 2008). Both Rictor and Sin1 knockout cells exhibited reduced ER-mitochondrial contact (Figure 11D, page 46; Fig-

ure 12B and E, page 47). Fourth, to measure ER-mitochondrial contact at higher resolution (Csordas et al., 2006; Simmen et al., 2005; de Brito and Scorrano, 2008), we analyzed electron microscopic images of livers from Rictor knockout mice and wild type littermates (Figure 12C-D, page 47). ER-mitochondrial contact sites were reduced $\mp$40% upon mTORC2 knockout although total mitochondrial and ER content per cell were unchanged (Figure 11E, page 46). Furthermore, the mitochondrial network was fragmented in primary Rictor knockout hepatocytes as compared to wild type hepatocytes (Figure 11F, page 46). Finally, we examined the role of mTORC2 in MAM dynamics in live cells. mTORC2 was required for insulin stimulated MAM formation (Figure 11G, page 46). Thus, mTORC2 mediates MAM integrity and overall mitochondrial morphology. Furthermore, our results suggest that insulin signaling controls both localization of mTORC2 to MAM and MAM integrity.

We next examined if mTORC2 mediates insulin stimulated MAM integrity via Akt and PACS2. PACS2 is phosphorylated by Akt and is important for MAM integrity (Simmen et al., 2005; Aslan et al., 2009). We found that overexpression of wild type PACS2, but not PACS2 mutated at the Akt phosphorylation site (PACS2-S437A), was able to suppress the defect in ER-mitochondrial contact observed upon Rictor knockdown (Figure 11H-I, page 46). We also observed that both constitutively active Akt (Akt-S473D) and wild type PACS2 restored MAM in cells in which mTOR was pharmacologically inhibited (Figure 12F, page 47). Thus, mTORC2 at MAM appears to control MAM integrity via Akt and PACS2.

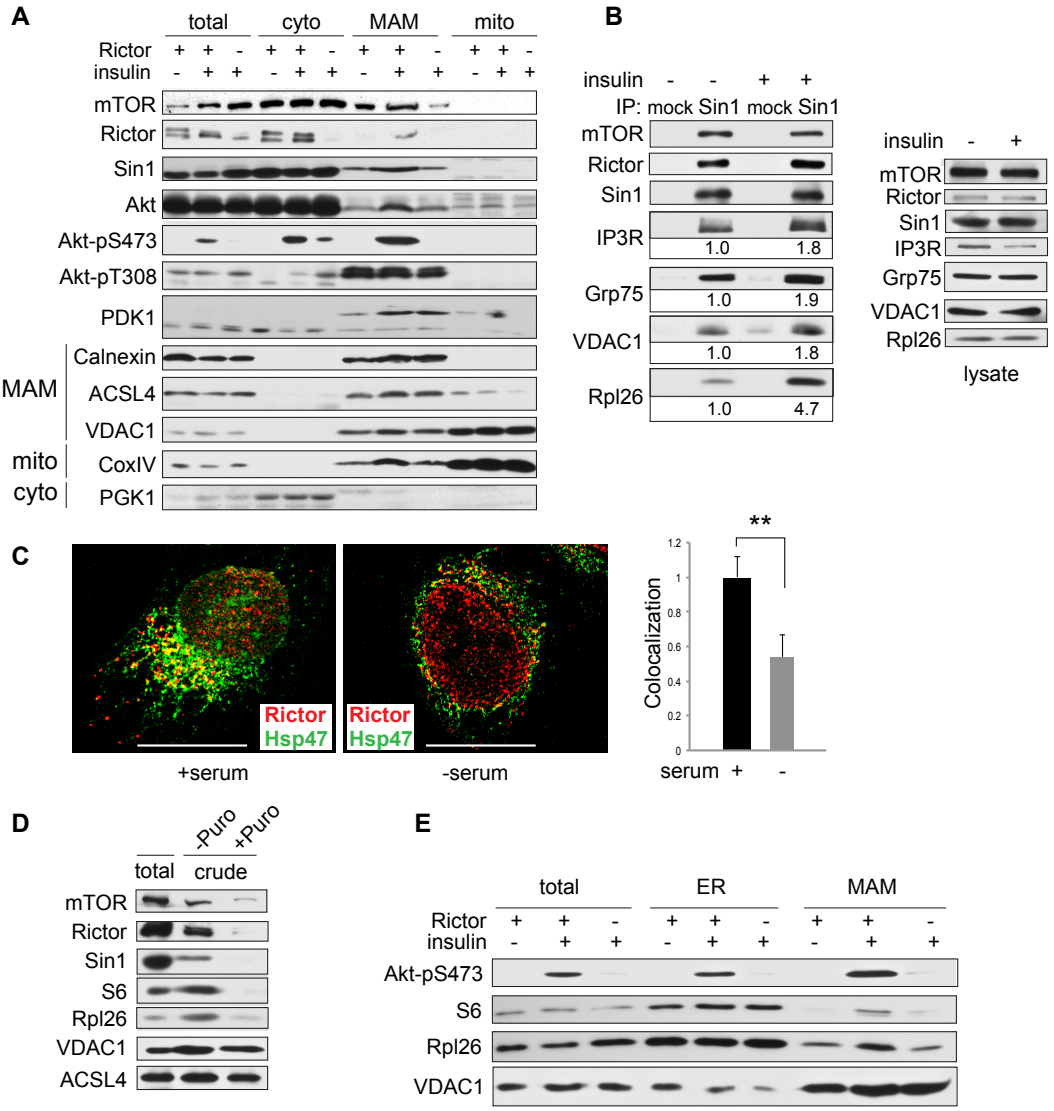

Figure 9: **mTORC2 - MAM localization is stimulated by growth factors and depends on mTORC2 - ribosome association. (A)** mTORC2 localization to MAM is stimulated by insulin. MAM was isolated from livers of Rictor knockout or control mice that were starved for 14h and injected with 4.5mg/kg insulin or saline 30 minutes prior to sacrifice. Lysates are pooled from 3 different mouse livers. **(B)** mTORC2-IP3R-Grp75-Rpl26 interaction is increased in total liver extracts from mice that were stimulated with insulin. Proteins were quantified relative to starved state. **(C)** mTORC2 - ER localization is reduced upon 14h serum starvation in U2OS cells. Pearson's correlation coefficient was calculated from confocal slices of individual cells and normalized to that of wild type cells (n=10). **(D)** mTORC2 MAM localization depends on ribosome interaction. Crude mitochondrial extracts from HeLa cells were treated with 1mM puromycin for 1h and repurified. Lysates illustrate loss of ribosomes and mTORC2 but not MAM components VDAC1 and ACSL4. **(E)** Mouse liver extracts show an mTORC2 dependent, growth factor induced increase of ribosomes at MAM. Lysates are pooled from 3 different mouse livers. Results are given as mean $\pm$ SEM. ($\star\star$ $p < 0.01$)

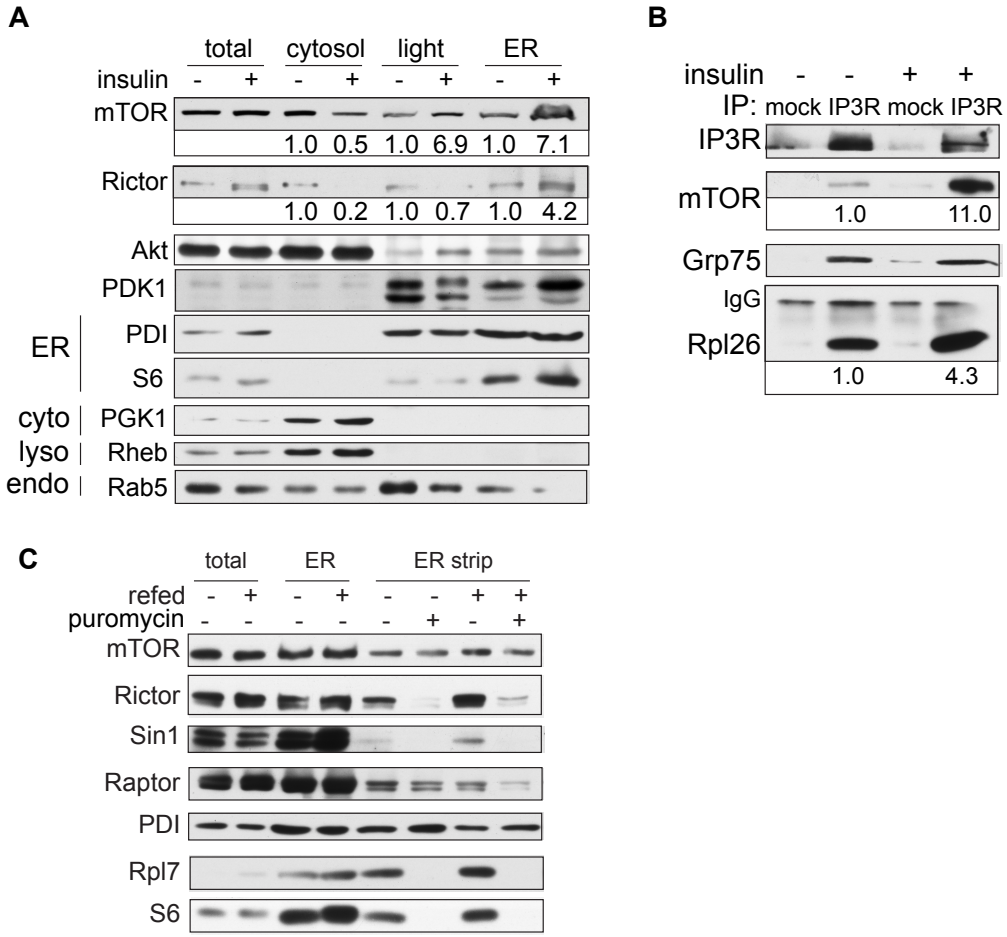

Figure 10: **Supplementary: mTORC2 - MAM localization is stimulated by growth factors and depends on mTORC2 - ribosome association. (A)** mTORC2 levels at ER are increased upon insulin stimulation. ER was isolated by isopycnic flotation from livers of mice that were starved for 14h and injected intra-peritoneally with insulin or saline. Intensities were quantified and averaged from 3 independent experiments and normalized to total levels and are relative to the starved condition. **(B)** mTORC2-IP3R-Grp75-Rpl26 interaction is increased after insulin stimulation. Immunoprecipitations were performed from total liver extracts of mice that were starved for 14h and injected intra-peritoneally with 4.5mg/kg insulin or saline 30 minutes prior to sacrifice. Proteins were quantified relative to starved state. **(C)** mTORC2 is associated to the ER via the ribosome. Rough ER from mouse livers was isolated and treated with 1mM puromycin in vitro for 1h, then repurified. Mice were starved for 14h, then refed on normal chow diet for 2h. Lysates were pooled from 3 different mouse livers.

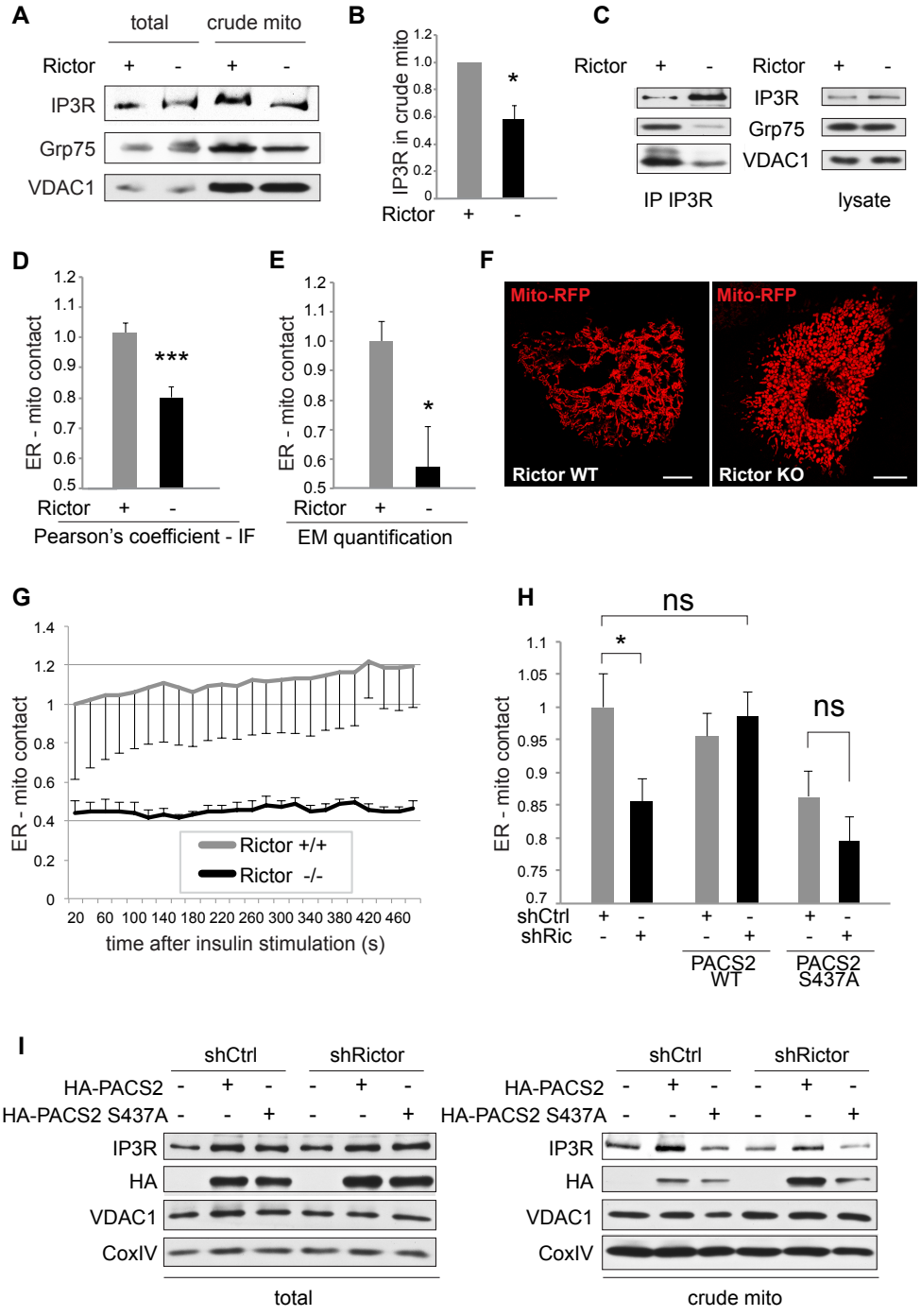

Figure 11: **mTORC2 maintains MAM integrity. (A)** Rictor KO MEFs show reduction of MAM proteins IP3R and Grp75 in crude mitochondrial extracts. **(B)** Quantification of IP3R from (A) (N=3). **(C)** MAM tether integrity is reduced upon Rictor KO. **(D)** Quantification of MAM from 3D IF images of MEFs by colocalization of GFP-ER and mRFP-Mito (N=9). **(E)** Quantification of MAM from EM pictures from mouse livers by visual colocalization of ER and mitochondria (N=6). **(F)** Mitochondrial network (Mito-RFP) of primary Rictor KO hepatocytes is fragmented. **(G)** Quantification of MAM formation after insulin stimulation (N=8). **(H)** MAM defect in Rictor knockdown HeLa cells is rescued by PACS2-WT but not PACS2-S473A) (N=10-16), measured by IF colocalization. **(I)** MAM defect in Rictor knockdown HeLa cells is rescued by PACS2-WT but not PACS2-S473A), measured by presence of IP3R in crude mitochondrial fraction. Results are shown as mean ± SEM

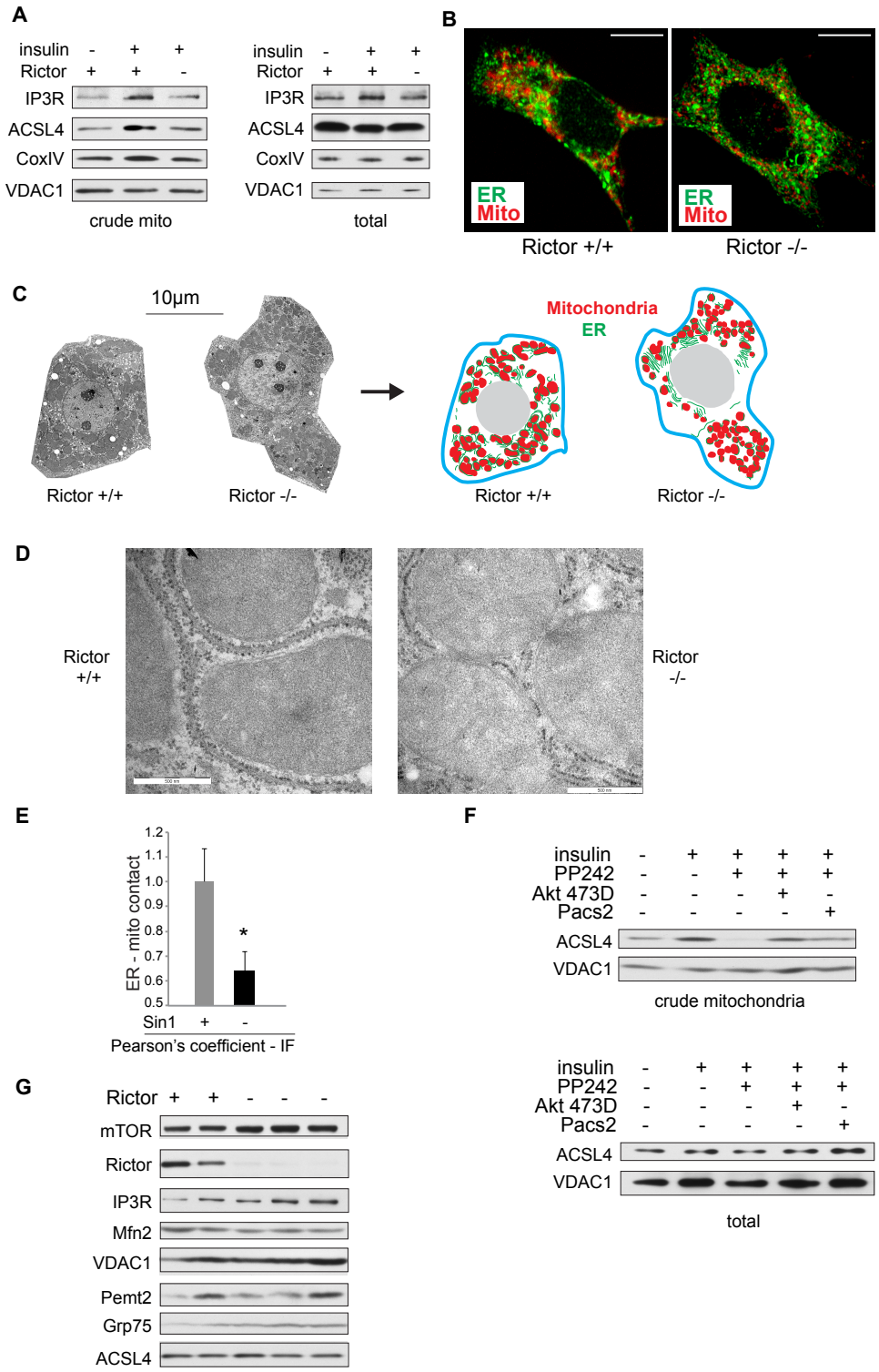

Figure 12: **Supplementary: mTORC2 maintains MAM integrity. (A)** Insulin stimulates MAM formation. Extracts from mouse livers. **(B)** Example of IF images used for quantification of MAM in Figure 11D. **(C)** Example of EM images from mouse livers used for quantification of MAM in Figure 11F. **(D)** Rictor KO MAM integrity is disrupted as judged from EM images of mouse livers. Bar = 500nm. **(E)** Quantification of ER-mitochondrial contact of Sin1 KO MEFs, measured by IF (N=4-5). **(F)** PACS2 and Akt S473D rescue MAM defect caused by mTORC2 inhibition by PP242 **(G)** No difference in total amount of MAM marker proteins upon Rictor KO in the liver of mice

mTORC2 controls MAM function

To examine further the role of mTORC2 at MAM, we investigated MAM function in mTORC2 deficient cells. Calcium flux between the ER and mitochondria occurs mainly at MAM (Patergnani et al., 2011; Bononi et al., 2012). We looked at ER/MAM calcium release upon stimulation with exogenously added ATP or upon pharmacological inhibition of SERCA pumps with thapsigargin (TG). Like MAM deficient cells (de Brito and Scorrano, 2008; Simmen et al., 2005; Bui et al., 2010), Rictor knockout MEFs displayed increased concentration of intracellular calcium $[Ca^{2+}]_i$ upon either ATP or TG treatment (Figure 13A-D, page 50). Furthermore, consistent with previous observations that Akt phosphorylates IP3R and thereby inhibits ER/-MAM calcium efflux (Khan et al., 2006; Szado et al., 2008; Marchi et al., 2008, 2012), we found that pharmacological inhibition of mTOR blocked IP3R phosphorylation (Figure 14D, page 51), and that overexpression of constitutively active Akt (Akt-S473D) suppressed the enhanced TG stimulated increase of $[Ca^{2+}]_i$ in mTORC2 deficient cells (Figure 13C-D, page 50). Thus mTORC2, via Akt, regulates calcium release at MAM.

As described previously in MAM deficient cells (de Brito and Scorrano, 2008), we also observed an increase in calcium uptake by mitochondria in mTORC2 knockout cells (Figure 13E, page 50 and Figure 14A-B, page 51). This is counter-intuitive as a reduction in calcium uptake by mitochondria might be expected in MAM or mTORC2 deficient cells. This seemingly paradoxical observation has been explained by the fact that MAM deficient cells contain larger ER calcium stores and thus release more calcium for uptake by mitochondria (de Brito and Scorrano, 2008). Accordingly, we also observed increased ER calcium stores in mTORC2 knockout primary hepatocytes and MEFs (Figure 13F, page 50 and Figure 14C, page 51). We note that enhanced calcium release at MAM can also be attributed to defective Akt, as shown above (Figure 14D, page 51).

mTORC2 controls mitochondrial physiology

Observations described above (Figure 11F, page 46 and Figure 13E, page 50) and elsewhere (see Introduction) suggest that mTORC2, like MAM (Contreras et al., 2010; de Brito and Scorrano, 2010), affects mitochondrial function. We examined mitochondrial function in mTOR complex 2 deficient cells. Rictor knockout cells exhibited an increase in routine (basal) respiration (Figure 15A, page 52), mitochondrial inner membrane potential ($\Delta\Psi_m$) (Figure 15B, page 52, Figure 16A-C, page 53) and ATP production (Figure 16E, page 53). The latter two effects were suppressed by overexpression of activated Akt (Akt-S473D) (Figure 15B, page 52; Figure 16B and E, page 53). Akt controls mitochondrial potential by phosphorylating hexokinase 2 (HK2) at T473

and thereby stimulating translocation of HK2 from the cytoplasm to the MAM protein VDAC1 (Miyamoto et al., 2008; Gottlob et al., 2001). We observed that mTOR inhibition blocked HK2-T473 phosphorylation in HeLa cells (Figure 15C, page 52). Furthermore, insulin stimulated recruitment of HK2 to mitochondria, likely MAM, in an mTORC2 dependent manner in HeLa cells, mouse liver, and MEFs (Figure 15D-E, page 52 and Figure 16D, page 53). Finally, we found that overexpression of mutant HK2 containing a phosphomimetic residue at position 473 (HK2-T473D) suppressed the increased mitochondrial potential observed in mTORC2 deficient cells (Figure 15F, page 52). Thus, mTORC2 controls mitochondrial energy production, at least in part via Akt mediated regulation of HK2 at MAM.

Mitochondria play a pivotal role in apoptosis. Arachidonic acid induces apoptosis by stimulating the transfer of calcium from the ER to mitochondria at MAM (Marchi et al., 2012; Rizzuto et al., 2009). Uptake of calcium by mitochondria can trigger apoptosis by inducing the mitochondrial permeability transition (MPT) (Gunter and Gunter, 2001). As described above, mTORC2 deficient cells display enhanced ER/MAM calcium release (Figure 13, page 50). Calcium release was also enhanced in Rictor knockout MEFs upon arachidonic acid, as compared to wild type MEFs (Figure 15G, page 52). Furthermore, an mTORC2 deficiency led to an increase in MPT (Figure 15H, page 52) and elevated levels of cleaved Parp (Figure 15I, page 52). As measured by Annexin V staining, pharmacologic inhibition of mTOR also caused an increase in apoptosis that could be suppressed by overexpression of wild type PACS2 (Figure 15J, page 52). Thus, mTORC2 at MAM appears to control several aspects of mitochondrial physiology.

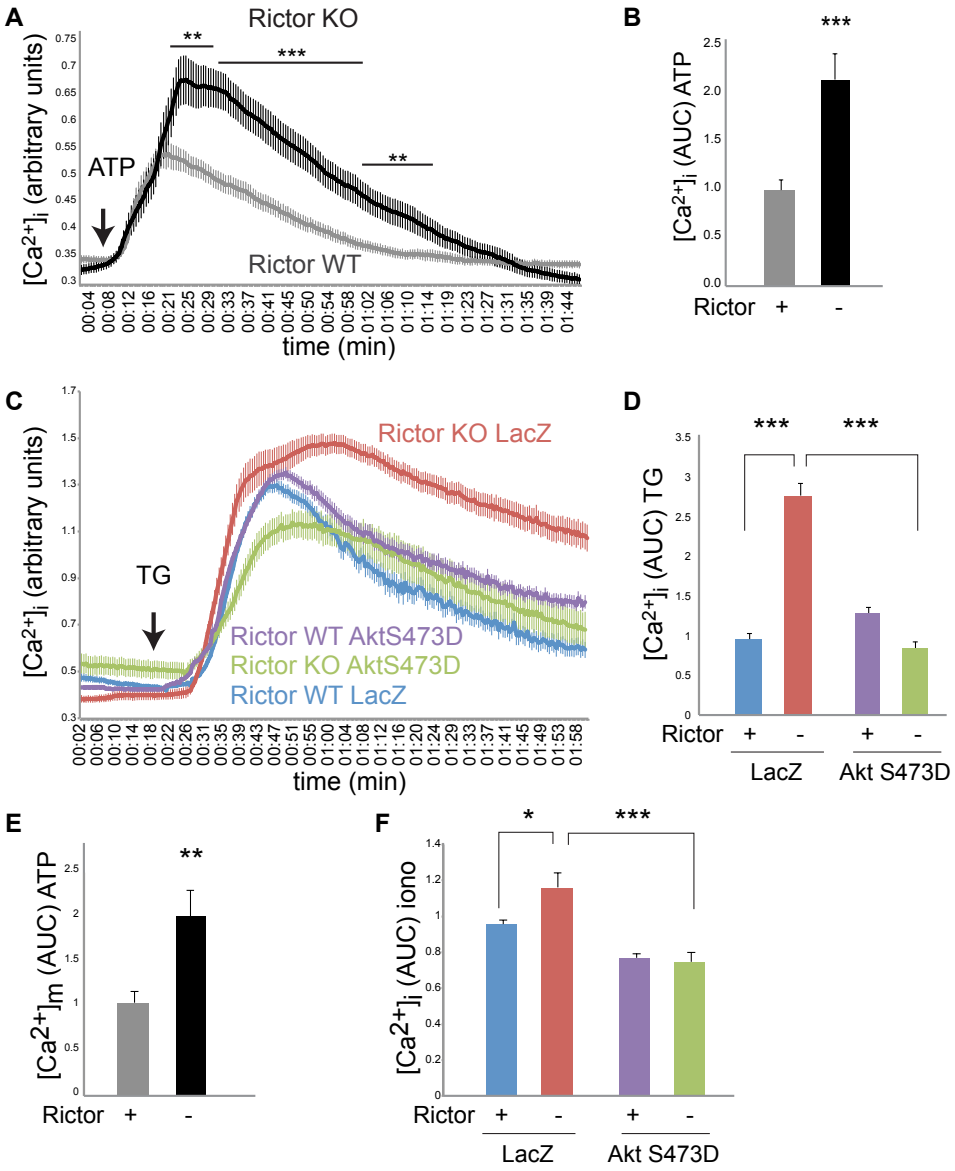

Figure 13: **mTORC2 controls MAM function. (A)** Intracellular calcium release in Rictor knockout and control MEFs after stimulation with 10µM ATP, visualized by Fura2-AM. **(B)** Area under the curve (AUC) of (A) (N=34). **(C)** Intracellular calcium release in Rictor knockout and control MEFs, expressing LacZ or Akt-S473D after stimulation with 10µM thapsigargin, visualized by Fura2-AM. **(D)** Area under the curve of (C) (N=16-54). **(E)** AUC of mitochondrial calcium increase in Rictor knockout and control MEFs after stimulation with 10µM ATP visualized by the cameleon probe 4mtD3cpv (N=20). **(F)** Area under the curve of intracellular calcium release in Rictor knockout and control MEFs, expressing LacZ or Akt S473D after stimulation with 10µM ionomycin visualized by Fura2-AM. Scale = arbitrary units. Results are shown as mean ± SEM and normalized to wild type cells. ($\star$ $p<0.05$, $\star\star$ $p<0.01$, $\star\star\star$ $p<0.001$)

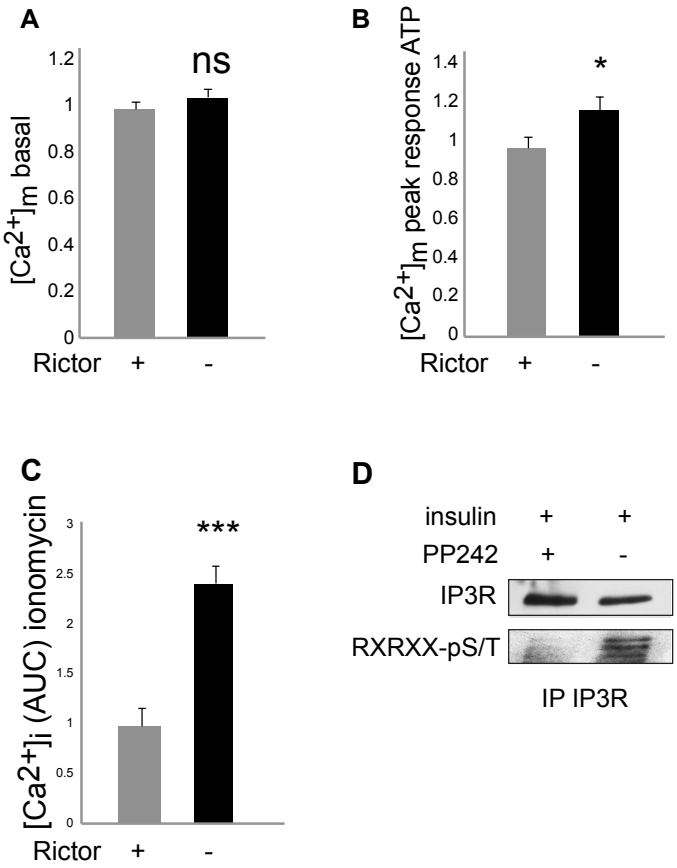

Figure 14: **Supplementary: mTORC2 controls MAM function. (A)** Basal mitochondrial calcium levels in Rictor knockout and control MEFs, arbitrary units (N=3). **(B)** Peak influx of mitochondrial calcium levels after ATP stimulation in Rictor knockout and control MEFs, arbitrary units (N=3). **(C)** Area under the curve of intracellular calcium release in Rictor KO and control primary hepatocytes after stimulation with 10μM ionomycin visualized by Fura2-AM (N=21-33), arbitrary units. **(D)** Phosphorylation of IP3R at Akt target site is sensitive to mTOR inhibition. HeLa cells were starved for 14h, treated with 500nM PP242 or DMSO for 20 minutes and stimulated with 20% FCS for 20 minutes. IP3R was immunoprecipitated and blots were probed with an anti-IP3R or an anti-phospho-Akt-Substrate (RXRXXpS/T) antibody. Results are shown as mean ± SEM and normalized to wild type cells. ($\star$p<0.05; $\star\star\star$ p<0.001)

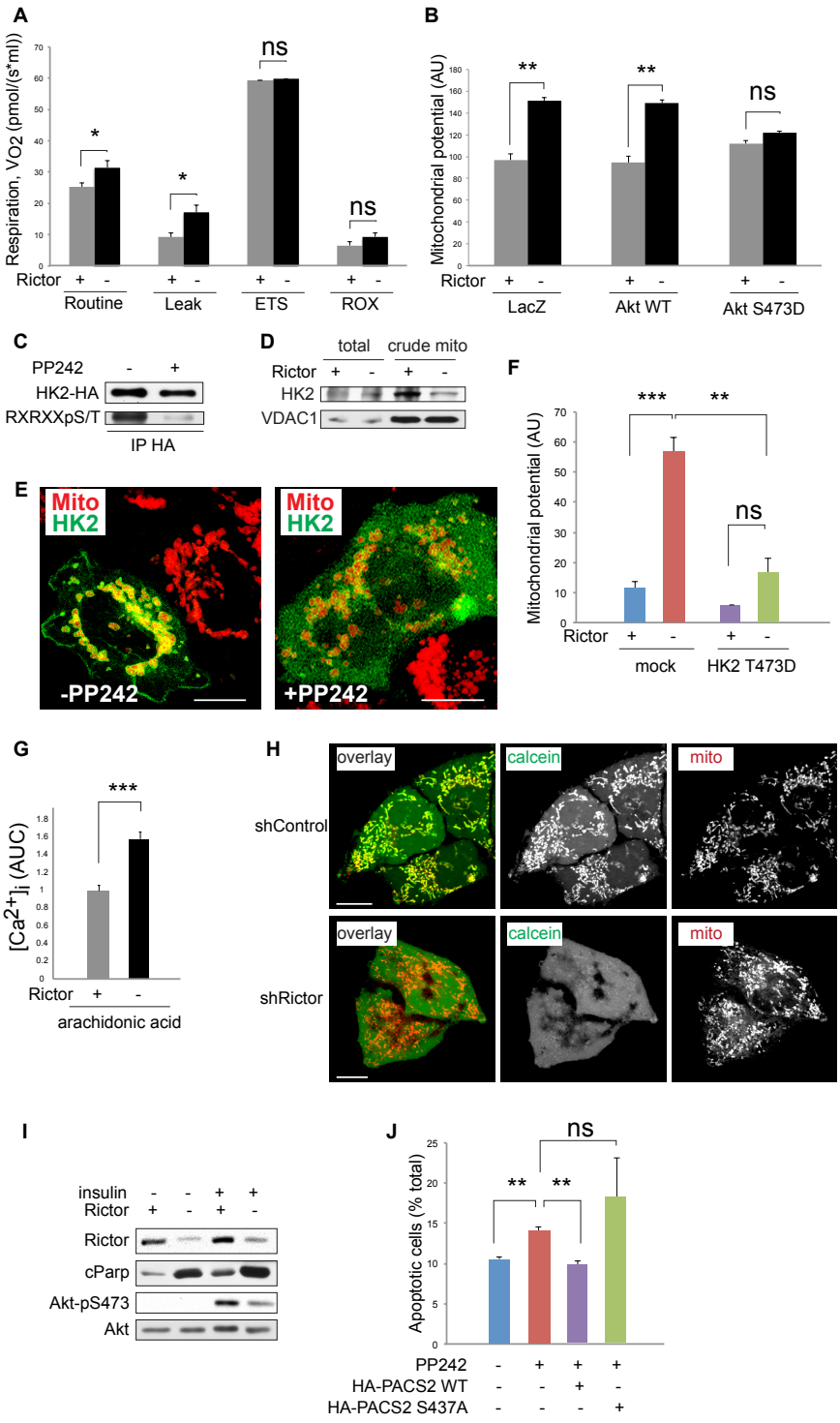

Figure 15: **mTORC2 controls mitochondrial function. (A)** Respiration of MEFs (N=4). **(B)** Mitochondrial potential of MEFs expressing LacZ, Akt-WT or Akt-S473D (N=3). **(C)** Akt mediated phosphorylation of HK2. **(D)** Localization of HK2 in MEFs. **(E)** GFP-HK2 localization to mitochondria is inhibited by mTOR inhibition (PP242). **(F)** Mitochondrial potential of Rictor KO MEFs is rescued by HK2 T473D (N=3). **(G)** AUC of intracellular calcium release of MEFs after treatment with 80µM arachidonic acid (N=67-70). **(H)** Mitochondrial pore permeability transition. Absence of mitochondrial calcein signal indicates permeability transition in Rictor knockdown HeLa cells. **(I)** Parp levels in MEFs. **(J)** Apoptotic HeLa cells expressing PACS2-WT or PACS2-S437A as determined by Annexin V staining (n=3).

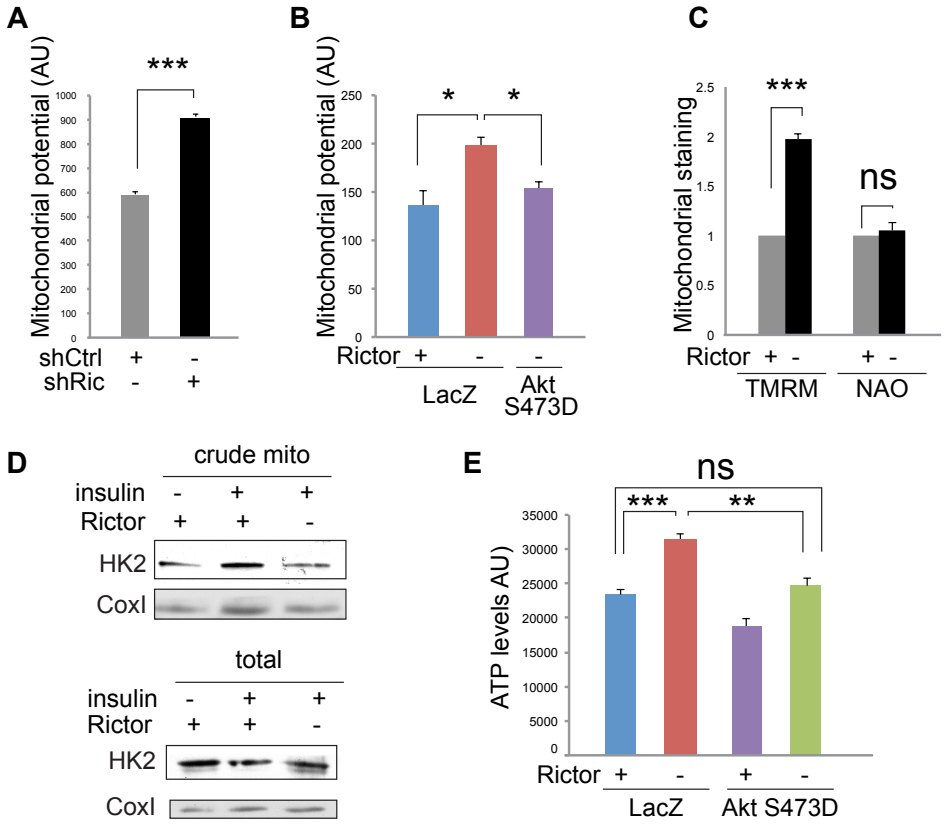

Figure 16: **Supplementary: mTORC2 controls mitochondrial function. (A)** Mitochondrial potential of shCtrl or shRictor expressing HeLa cells (N=3). **(B)** Mitochondrial potential of Rictor knockout or control primary hepatocytes expressing either LacZ or Akt-S473D (N=3). **(C)** Mitochondrial staining intensity of Rictor knockout and control MEFs, measured by mitochondrial potential dependent (TMRM) or independent staining (NAO) (N=3). **(D)** Crude mitochondrial extracts of mouse liver extracts analyzing localization of HK2. **(E)** ATP levels of Rictor knockout and control MEFs infected adenovirally with LacZ or Akt-S473D, measured by CellTiter-Glo luminescence assay (N=12). Results are shown as mean $\pm$ SEM and normalized to wild type cells. ($\star$ p<0.05; $\star\star\star$ p<0.001)

2.1.4 *Discussion*

We show that mTORC2 is physically associated with MAM, and that mTORC2-Akt signaling mediates MAM integrity and function. First, mTORC2 localizes to MAM in a growth factor stimulated manner. Second, mTORC2 is required for MAM integrity and function, at least in part through Akt phosphorylation of PACS2, IP3R and HK2. Third, via its role in MAM, mTORC2 also affects ER and mitochondrial functions, in particular calcium flux and energy production. Accordingly, we find that mTORC2 disruption phenotypically mimics a MAM deficiency. Thus, MAM appears to be an mTORC2 signaling hub.

mTORC2 is activated by association with the ribosome and thereby directly couples the growth potential of the cell, i.e. the ribosome pool, to the control of cell growth and survival (Zinzalla et al., 2011). We find that mTORC2 exerts this control at least partly at MAM. Electron microscopy studies (Csordas et al., 2006; Lebiedzinska et al., 2009; RUBY et al., 1969) and proteomic profiling (Zhang et al., 2011; Poston et al., 2011) of MAM suggest that ribosomes are present at this ER subdomain, although the function of MAM localized ribosomes is unclear. Zhang *et al.* (Zhang et al., 2011) reported an increase in the number of ribosomes at MAM after CMV infection, a virus that is known to activate mTORC2 (Clippinger et al., 2011). Finally, calnexin, a MAM enriched chaperone (Lynes et al., 2011; Myhill et al., 2008), interacts with PACS2 and anchors ribosomes at the ER and possibly MAM (Lakkaraju et al., 2012; Chevet et al., 1999). It remains to be determined whether calnexin is indeed involved in localization of ribosomes to MAM or in mTORC2 signaling.

The presence of mTORC2 and phosphorylated Akt at MAM suggests that mTORC2 phosphorylates Akt (S473) at MAM. Akt is generally thought to be localized to the plasma membrane, upon binding of its PH domain to PIP_3, where it is phosphorylated by PDK1 at T308 and by mTORC2 at S473. However, previous studies have reported active Akt at mitochondria and MAM (Giorgi et al., 2010; Antico Arciuch et al., 2009; Miyamoto et al., 2008; Bijur and Jope, 2003). Furthermore, insulin stimulation induces translocation of phosphatidylinositol 3-kinase (PI3K), the enzyme that catalyzes PIP_3 formation, from the plasma membrane to the ER (Daniele et al., 1999). Finally, we find PDK1 and T308 phosphorylated Akt at MAM (Figure 9A, page 44). Thus, there appears to be spatially distinct mTORC2 signaling pathways. Interestingly, there are at least three different mTORC2s containing a distinct Sin1 isoform (Frias et al., 2006).

Here we report that hepatocytes from liver specific Rictor knockout mice are defective for MAM. Hagiwara *et al.* recently reported that Rictor knockout mice have whole body metabolic defects (Hagiwara et al., 2012). Interestingly, liver specific knockout of Mfn2, a key MAM protein, confers a whole body phenotype strikingly similar to

that of liver specific Rictor knockout. Both knockout mice exhibit increased gluconeogenesis, hyperinsulinemia and glucose intolerance (Sebastián et al., 2012). This strong metabolic phenotype is consistent with the fact that the liver relies heavily on MAM function and that MAM mediates glucose metabolism (Rusinol et al., 1994; Zhang et al., 2011). The mTORC2 deficient liver is also defective in lipogenesis due to a failure in Akt dependent activation of SREBP1c (Hagiwara et al., 2012). It is conceivable that mTORC2 regulated MAM integrity is important for the activation of SREBP1c, as ceramide synthesis affects SREBP translocation and as MAM is important for ceramide metabolism (Bionda et al., 2004; Worgall et al., 2004). The reduction in liver weight upon mTORC2 knockout (Hagiwara et al., 2012) can be explained by an increase in apoptosis due to a MAM deficiency. Furthermore, a previously reported whole genome shRNA screen revealed that mTORC2 addicted cancer cells are dependent on mitochondrial function and in particular HK2 (Colombi et al., 2011). Thus, MAM appears to be a particularly important hub for mTORC2 signaling in the control of growth and metabolism.

In summary, we demonstrate that mTORC2 can localize to MAM, where it controls growth factor mediated MAM integrity, calcium flux, and mitochondrial physiology. mTORC2 controls these processes via Akt, which in turn regulates PACS2, IP3R and HK2 at MAM. The combined action of these substrates affects energy metabolism and cell survival. While the role of MAM localized mTORC2 in disease remains to be clarified, it is tempting to speculate that there could be beneficial effects of novel therapeutic interventions that specifically target mTORC2 localized to MAM.

2.1.5 *Material and Methods*

Cell culture

HeLa and inducible Rictor knock-out MEFs were cultured, transfected, stimulated and harvested as described previously (Jacinto et al., 2004; Cybulski et al., 2012; Thedieck et al., 2007). Briefly, cells were seeded and grown for 48 hours in DMEM supplemented with 10% serum (basal conditions). Cells were starved of serum for 14 hours unless noted otherwise before re-stimulation with 100nM insulin (Sigma) for 10 minutes.

Primary hepatocytes: Cells were isolated using previously described protocol (Hagiwara et al., 2012).

mTORC2 knockdown: HeLa cells were infected with lentiviruses carrying shControl or shRictor (TRC library, Sigma Aldrich) at MOI of 0.1. Cells were selected after 24h with 1µg/ml puromycin for 1 week, then subcloned and kept under puromycin selection.

Transfection: Transfection was performed using X-tremegene HD (Roche) using per 10cm dish 1ml Optimem (Gibco), 6µg plasmid DNA and 18µl transfection reagent.

Plasmids

GFP-tagged HK2 was kindly provided by Alenoush Vartanian. HA-tagged mouse HK2 (HA-HK2) in pcDNA3.1 was kindly provided by Nobuyuki Tanaka (Ando et al., 2010). HK2-T473D was generated from HA-HK2 by mutagenesis (Zheng et al., 2004) using the following primers GAAGgacCTGGAGTCTCTGAAGC and CAGgtcCTTCTGGCGGGCCC. Mutagenesis was sequence verified. Adenoviral LacZ, Akt-WT and Akt-S473D were produced as previously described (Hagiwara et al., 2012). PACS2-HA and PACS2-S437A-HA (Aslan et al., 2009) were obtained from Prof. Gary Thomas. 4mtD3cpv was kindly provided by Dr. Amy Palmer and Roger Tsien.

Protein lysates, immunoprecipitations

Protein extracts were prepared as previously described (Thedieck et al., 2007; Jacinto et al., 2004), resolved on SDS-PAGE and transferred to nitrocellulose membranes (Protran, Whatman). Immunoprecipitation and immunoblotting were performed as previously described (Thedieck et al., 2007; Jacinto et al., 2004; Zinzalla et al., 2011). Subcellular fractions were normalized to protein concentration prior to loading. Films were scanned on CanoScan 9000F and signals were quantified using densitometry functions of the FIJI application. Percentage of different proteins in a given fraction were calculated as protein densitometry multiplied by volume and protein concentration of that fraction divided by the amount in the total extract.

Subfractionation: ER was isolated by isopycnic flotation using previously described protocol (Stephens et al., 2008; Lerner et al., 2003). Crude and pure mitochondrial extracts, MAM isolation: Performed as previously published (Wieckowski et al., 2009).

Ribosome stripping: Experiment was performed as previously published (Adelman et al., 1973).

Phosphorylation status of IP3R: HeLa cells were starved for 14h, treated with 500nM PP242 or DMSO for 20 minutes and stimulated with 20% FCS for 20 minutes. IP3R was immunoprecipitated and blots were probed with anti-IP3R or anti-phospho-Akt-Substrate antibody.

Phosphorylation status of HK2: HeLa cells were transfected with HK2-HA 48h prior to experiment. HeLa cells were starved for 14h, treated with 500nM PP242 or DMSO for 20 minutes and stimulated with 20% FCS for 20 minutes. Blots were probed with anti-HA or anti-phospho-Akt-Substrate antibody.

Insulin stimulation of mice: Mice were starved for 14-16h over night, then injected IP with 4.5mg/kg insulin or saline 30 minutes prior to sacrifice. Refed mice were starved for 14-16h over night, then refed a standard chow diet for 2h.

Microscopy

Immunofluorecence: For organelle labeling experiments, cells were infected 24h prior to harvest with baculovirus expressing ER-GFP or Mito-RFP (BacMAM, Life Technologies). Cells were seeded on 12mm glass slides 24h before experiment. Cells were washed in PBS, fixed with 37°C 4% paraformaldehyde for 2 minutes, washed in PBS 2 times, treated with 0.1% Triton X100 for 10 minutes at room temperature, blocked in 3% BSA for 1h at room temperature. Primary antibodies were added over night at 4°C (1:50 for anti-rictor, 1:200 for anti-HSP47), washed 3 times in PBS at room temperature and incubated with secondary antibodies (anti-rabbit-Alexa647 1:200, anti-mouse-Alexa488 1:200) for 2h at room temperature. Slides were washed 3 times in PBS and mounted in Mowiol mounting medium (2.4g Mowiol 4-88 Calbiochem, 6g glycerol, 6ml water, 12ml 0.2M Trix pH8.5, 2.5% w/v [1,4,-diazobicycli-[2.2.2]-octane Sigma-Aldrich). Fluorescence was measured on LSM510 or LSM710. Colocalization (Pearson's correlation coefficient) was measured with Imaris application using automatic thresholding.

Electron microscopy: Embedding protocol: Mouse liver was cut into small pieces of 1-2 mm and fixed with 0.1M phosphate buffer containing 3% formaldehyde and 0.3% glutaraldehyde for 30min at RT and with fresh fixative overnight at 4°C.Then samples were washed with PBS 3x30min. Dehydratation was done at 4°C in 50%,70%,90% methanol/PBS each for 1h. Infiltration with LR gold was done according to the manufacturer's instructions (LR gold London Resin company). Polymerization was done at -10°C for 24h. Sections of 75nm were collected on Formvar/ carbon coated Ni-grids. Sections were stained for 15min with 4% uranyl-acetate and contrasted with lead-citrate for 60sec., visualized by a Philips EM100 electron microscope. Images were cropped for individual hepatocytes using Adobe Photoshop. Cell parameters including ER length and mitochondrial size were calculated using FIJI application.

Statistical Analyses

Data were expressed as average $\pm$ SEM of at least three independent experiments. Unpaired t test was used to determine differences between two groups. Significance was judged when $p < 0.05$.

Animals

Mice were generated as previously described (Hagiwara et al., 2012). All experiments were performed in accordance with federal guidelines and were approved by the Kantonales Veterinäramt of Kanton Basel-Stadt.

Chemicals

Antibodies: mTOR, Akt, Akt pS473, Akt pT308, S6, PDI, Insulin receptor, Lamp1, Rictor IB, VDAC1, IP3R, Grp75, PDK1, Rheb, HA, Mfn2, RXRXXpS/T (9611), HK2, CoxI, cleaved Parp from Cell Signaling; Sin1, Raptor, Rpl26 from Bethyl; PGK1, ACSL4, from Santa Cruz; PEMT2, Rictor IF from Protein Atlas; Rab32 from Sigma; Calnexin from BD Bioscience; CoxIV from Invitrogen; Hsp47 from Enzo Life Science; Rictor EM from Abcam (ab104838); Rab5 antibody was obtained from Martin Spiess; Rictor IP antibody was obtained from Markus Rüegg.

Chemicals: PP242 (Chemdea); insulin, ionomycin, thapsigargin and arachidonic acid (Sigma); Fura 2 AM (Invitrogen).

Metabolic measurements

Mitochondrial potential measurements were performed using MitoPT - TMRM (ImmunoChemistry Technologies LLC) following the manufacturers' instructions on a Biorad Biomek NK and statistical analysis was performed using FlowJo 9.4.

Permeability transition: Calcein staining was performed using the Image-iT LIVE Mitochondrial Transition Pore Assay Kit (I35103) (Invitrogen) following recommended protocol.

Mitochondrial Ca^{2+}: MEFs were transiently transfected with the ratiometric cameleon probe 4mtD3cpv targeted to the mitochondrial matrix 48h before the experiments. Ratiometric images of Ca^{2+} signals were obtained using a microscope (Axio Observer, from Zeiss) equipped with the Lambda DG4 illumination system (Sutter Instrument Company, Novato, CA, USA). Cells were illuminated at 440 nm (440AF21; Omega Optical) through a 455DRLP dichroic mirror, and emission was collected alternatively at 480 nm (480BP10; Omega Optical) and 535 nm (535AF26; Omega Optical), using a cooled, 12-bit CCD camera (CoolSnap HQ, Ropper Scientific, Trenton, NJ, USA). Image acquisition and analysis were performed with the Metafluor 6.3 software (Universal Imaging, West Chester, PA, USA). Experiments were performed at room temperature in Hepes-buffered solution containing (in mM): 135 NaCl, 5 KCl, 1 MgCl2, 1 EGTA, 10 Hepes, 10 glucose, pH adjusted at 7.45 with NaOH.

Intracellular Ca^{2+}: Cells were plated 48h before experiment on Lab-Tek chamber slides (Thermo). Chambers were washed loaded for 30

minutes with HBSS plus calcium, 10% FCS and 1µM Fura2 AM (Invitrogen). 10 minutes before measurement and medium was replaced by adding calcium free HBSS (Sigma) and Fura-2 signal was measured as described previously (de Brito and Scorrano, 2008). Signal was captured and processed on Zeiss Cell Observer light microscope. Area under the curve was calculated with Graphpad Prism 5 using baseline correction. Stimulations were performed with 10µM ATP, 10µM thapsigargin, 80µM arachidonic acid or 10µM ionomycin.

To measure the oxygen consumption in MEF cells, we used the Oxygraph-2k (Oroboros Instruments). After centrifugation and resuspension in IMDM medium (Sigma-Aldrich) without any additions, the measurement of Rictor wildtype MEFs and Rictor knockout MEFs was performed in parallel chambers using 2 million cells per cell line. Following inhibitors and uncouplers have been used: Oligomycin at 2µM; FCCP at 1µM; Rotenon at 0.5µM (manufacturer); Antimycin A at 2.5µM (manufacturer). Routine, leak, ETS and ROX were calculated as described before (Gnaiger, 2008).

Apoptosis: Annexin V Alexa Fluor 488 (Invitrogen) measurements were performed as previously described (Colombi et al., 2011).

ATP: ATP was quantified using CellTiter-Glo Luminescent assay (Promega).

Statistics: Student's T Test was calculated using QuickTTestX 1.0 application.

2.1.6 *Acknowledgements*

We thank Asami Hagiwara and Dr. Marion Cornu for the generation and maintenance of the transgenic mice. We acknowledge Cyril Castelbou for technical support. We thank Dr. Don Benjamin for his careful and critical reading of the manuscript. We acknowledge support from the Leslie Misrock Foundation (C.B), the Swiss National Science Foundation, SystemsX.ch, the Swiss Cancer League, the Louis Jeantet Foundation, and the Canton of Basel. We declare that no competing interests exist.

2.2 ADDITIONAL RESULTS

The following sections deal with supplementary findings that could not be included in the manuscript. Some of the data presented here are preliminary and should be regarded as starting points for further investigations into the role of mTORC2 at MAM.

2.2.1 *mTORC2-ribosome-enriched mRNAs*

2.2.1.1 *Introduction*

As mentioned previously, ribosomes bind mTORC2 and are important for its activation (Zinzalla et al., 2011). As there are many more ribosomes than mTORC2 in a cell, we asked whether mTORC2 associates only with a subset of ribosomes. It should be noted that these results came chronologically before the discovery of mTORC2 localization to the ER/MAM. This project initially followed the study of the upstream mechanisms governing ribosome mediated mTORC2 activation. However at a later stage, this interesting aspect, that still deserves further study, was not followed up on and we instead focused more on the downstream function of mTORC2-MAM localization.

Ribosomal heterogeneity is a term that was first used in the 1970s to describe different functional classes of ribosomes defined by different structural composition (Bickle and Howard, 1973). Newer studies suggest that structurally different ribosomes can translate different mRNAs (Mauro and Edelman, 2002; Komili et al., 2007). On a more fundamental level, ribosomes are classified into ER-translating or cytosolic ribosomes. ER associated ribosomes are generally thought to be structurally identical to cytosolic ribosomes. The emergence of the translated signal peptide recruits the signal recognition particle (SRP). This leads to the translocation of the message into the ER lumen. Some reports also suggest the possibility of SRP-independent translation at the ER (Potter and Nicchitta, 2000). After termination of translation, fully assembled ribosomes can stay bound at the ER and initiate translation of secreted and cytosolic proteins (Adelman et al., 1973; Potter and Nicchitta, 2000). It is estimated that roughly 50%-90% of the cellular ribosomes are associated to the endoplasmic reticulum, depending on the cell type and growth conditions (Stephens et al., 2005).

2.2.1.2 *Results*

To identify if mTORC2 is associated with a subset of ribosomes, we performed a microarray-based study to identify mRNAs that either co-purified with whole ribosomes or with mTORC2-associated ribosomes. We assumed that even though mTORC2 is activated by association to ribosomes in a translation-independent manner, the nature

of these potentially enriched messages could help elucidate mTORC2 activity.

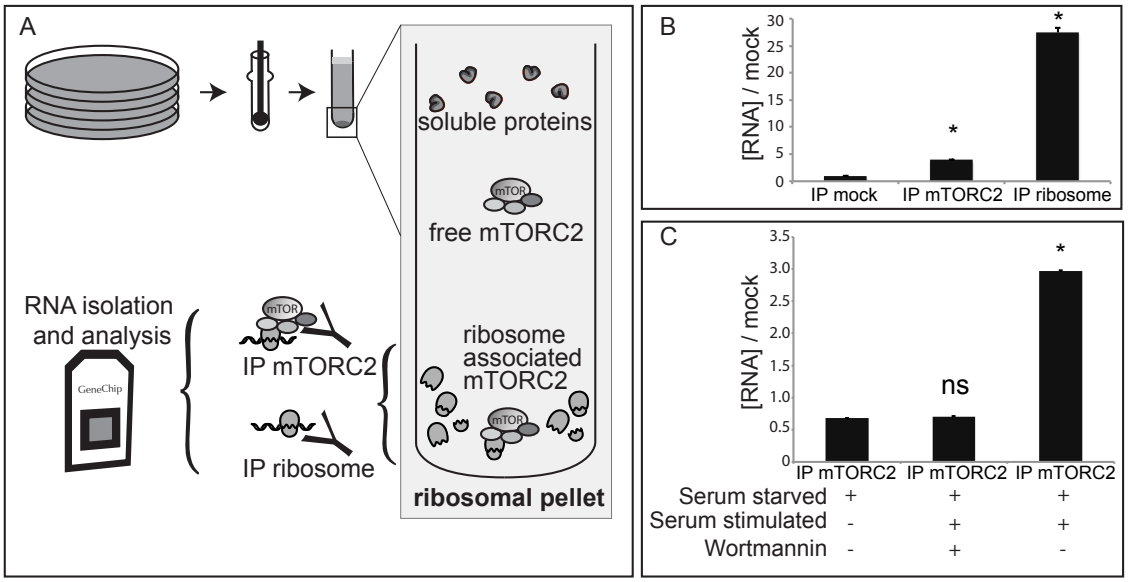

Figure 17: **Purification of mTORC2 - associated mRNAs. (A)** Schematic representation of the working protocol. Cells are homogenized, and lysates are centrifuged through a sucrose cushion. Ribosomal pellets including ribosome associated mTORC2 are recovered and used for immunoprecipitations. **(B)** Amount of RNA recovered from mTORC2 and ribosome immunoprecipitations, normalized to mock IP. **(C)** Quantification of RNA recovered from an mTORC2 immunoprecipitation under different growth conditions.

Ribosome-associated mTOR complex 2 can be purified from ribosomal pellets (Zinzalla et al., 2011). To specifically enrich for ribosome-associated mTORC2, we performed immunoprecipitations from purified ribosomal lysates of mTORC2 (Sin1) or of ribosomes (Rpl26) (Figure 17A, page 62). First, we quantified the amount of RNA co-purified in mTORC2 or ribosome immunoprecipitations relative to a mock IP (Figure 17B, page 62). Immunoprecipitation of mTORC2 co-purified 4 times more RNA than a mock IP, and immunoprecipitation of ribosomes co-purified 27 fold more RNA than the mock IP. Second, we performed the experiment after serum starvation and restimulation in presence or absence of the PI3K inhibitor wortmannin. We find a PI3K-dependent increase of co-immunoprecipitated RNA and thus mTORC2 ribosome interaction under serum stimulation (Figure 17A, page 62). These observations are in agreement with previous studies (Zinzalla et al., 2011).

To identify potentially enriched or excluded mRNAs from mTORC2-ribosomes, we loaded an equal amount of co-purified mRNA from either mTORC2-IP or ribosome-IP on Affymetrix microarrays. 6740

Annotation Cluster 1	Enrichment Score: >150				
Category	Term	Count	%	PValue	Bonferroni
SP_PIR_KEYWORDS	transmembrane	1198	73	<1.0E-250	<1.0E-250
SP_PIR_KEYWORDS	membrane	1257	76	<1.0E-250	<1.0E-250
UP_SEQ_FEATURE	transmembrane region	1196	73	<1.0E-250	<1.0E-250
GOTERM_CC_FAT	GO:0016021~integral to membrane	1217	74	1.1E-215	4.6E-213
GOTERM_CC_FAT	GO:0031224~intrinsic to membrane	1233	75	5.6E-211	2.4E-208
Annotation Cluster 2	Enrichment Score: 112.91				
Category	Term	Count	%	PValue	Bonferroni
SP_PIR_KEYWORDS	endoplasmic reticulum	330	20	2.2E-166	1.2E-163
GOTERM_CC_FAT	GO:0005783~endoplasmic reticulum	390	24	5.4E-118	2.3E-115
GOTERM_CC_FAT	GO:0044432~endoplasmic reticulum part	162	10	1.49E-56	6.41E-54
Annotation Cluster 3	Enrichment Score: 109.02				
Category	Term	Count	%	PValue	Bonferroni
SP_PIR_KEYWORDS	signal	725	44	1.1E-170	6.2E-168
UP_SEQ_FEATURE	signal peptide	725	44	7.7E-170	2.6E-166
SP_PIR_KEYWORDS	disulfide bond	473	29	2.66E-52	1.5E-49
UP_SEQ_FEATURE	disulfide bond	447	27	3.7E-46	1.25E-42

Figure 18: **Cluster analysis of mTORC2 enriched mRNAs**. mRNAs found enriched on mTORC2 ribosomes were analyzed for functional clustering by the online application DAVID. Three clusters with highest scores are shown.

messages of a p-value <0.05 could be identified in both immunoprecipitates and the ratio of the mRNA signal from the mTORC2 IP divided by the signal from the ribosome IP was plotted on a histogram (Figure 19B, page 64). As expected, a majority (72%) clustered around a ratio of 1, meaning that they were equally likely to be found on an mTORC2-associated ribosome as any other ribosome. Of special interest to us were the mRNAs enriched more than 1.5 fold, which comprised 1770 messages (26%).

We analyzed this list of mTORC2-ribosome enriched mRNAs with the online tool DAVID (The Database for Annotation, Visualization and Integrated Discovery v6.7) and found among the highest enrichment clusters the terms "transmembrane" (72% of the enriched messages), "endoplasmic reticulum" (24%) and "signal peptide" (44%). All these terms are a common feature of endoplasmic reticulum translated mRNAs (Figure 18, page 63). These results were further confirmed by qPCR validation (Figure 19B, page 64).

Of note, there was only a minority of mRNAs excluded from mTOR complex 2-associated ribosomes (2%), containing among others a cluster of cytosolic proteins. Whether this unequal distribution of enriched / excluded mRNAs reflects a biological phenomenon such as the translation of cytosolic proteins at the endoplasmic reticulum (Lerner et al., 2003) remains unknown.

We also analyzed the mTORC2-enriched mRNAs for any motifs that might be enriched in their respective UTRs. These mRNAs were enriched in UNR binding sites (1.8 fold enriched, UNR complex = assembly platform for formation of decay mRNA-protein complex), K-

box, GY-box and BRD-box (2.4 fold enriched, miRNA seed sequences enriched in Notch target genes) and IRES (1.4 fold enriched, internal ribosome entry site). The significance of these motifs in the context of mTORC2 is unknown.

Complete dataset in Excel format will be available online under http://edoc.unibas.ch .

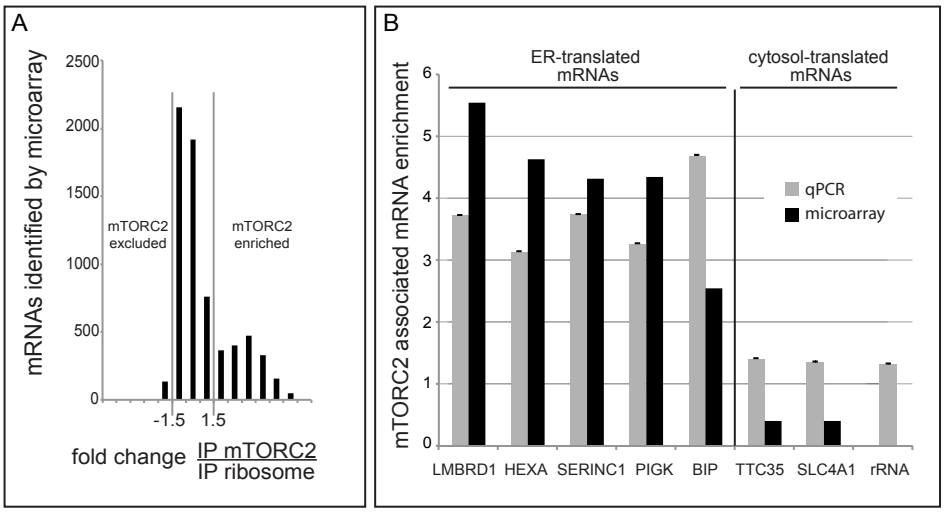

Figure 19: **mTORC2 - ribosome enriched mRNAs (A)** Histogram of the ratios of the mRNA signal from an mTORC2 ribosome divided by the signal of that same mRNA from a ribosome IP. Messages are considered mTORC2-ribosome enriched/excluded if their ratios exceed 1.5 fold change. **(B)** Confirmation of microarray data by qPCR. Ratios of ER and cytosol translated mRNAs were checked in immunoprecipitates from independent experiments. Grey bars indicate results from qPCR, black bars indicate original ratios from the microarray experiment.

2.2.1.3 *Discussion and outlook*

We show that mRNAs that code for ER translated messages can be co-purified from mTORC2-associated ribosomes. Our results confirm that mTORC2 associates to ribosomes in a growth factor-PI3K regulated manner. Furthermore, these data provide more evidence for mTORC2 localization to the ER and mTORC2-ribosome interaction at the ER.

Chronologically, these were the first data that indicated mTORC2 presence at the ER, even before our biochemical approaches (Figure 7B, page 40) or an independent study (Boulbés et al., 2011) confirmed this localization. In the spirit of generating a more focused manuscript, we decided to omit this part and present it here.

One question is how do these data fit in with MAM localization of mTORC2. mTORC2 localizes to MAM (Figure 7F, page 40), and is bound to ribosomes at this site (Figure 9D, page 44). Furthermore,

we found that ribosomes move to MAM upon insulin stimulation in a growth factor dependent manner (Figure 9E, page 44). Very little information is available on ribosomes and their function at MAM. Initially, it was thought that MAM forms only at the smooth ER, but as discussed earlier, these claims have been refuted. A functional role for ribosomes at MAM might exist in the context of spatially regulated translation, similar to local translation that is observed at neuronal synapses (Wang et al., 2010a). It is up to speculation as to what these locally translated messages might be, however it is interesting to note that some viruses such as the cytomegalovirus (that is also know to affect mTORC2 signaling) change the composition of MAM and actively recruit the translation machinery to MAM, by an unknown mechanism (Zhang et al., 2011). A hypothetical example for MAM restricted translation could be mitochondrial proteins, as the co-translational import of proteins into mitochondria has been suggested previously (Ahmed and Fisher, 2009). It should be noted though that there were no obvious clusters in the mTORC2 enriched mRNAs that might point to localized translation of MAM or mitochondrial proteins.

Several more steps could be investigated to complete this part of the project. One open question for example is if mTORC2 is involved in the translation of the mTORC2-ribosome enriched mRNAs. We analyzed some potential candidates for their respective protein levels and found no difference in liver-specific mTORC2 knockout lysates compared to wild type littermates (data not shown). As noted earlier, this does not exclude the possibility that mTORC2 is involved in regulating local translation of these messages.

It would be interesting to isolate ribosomes and their respective mRNAs specifically from MAM. This could be done in a growth factor dependent manner in combination with knocking out mTORC2. mRNAs could then be normalized to total mRNAs in order to look for any locally translated messages at MAM.

One unknown factor is the sequence of events leading to mTORC2 binding to the ER-translating ribosomes. Two possibilities exist: 1) mTORC2 binds these ribosomes in the cytoplasm and then moves along to the ER (e.g. by interacting with the SRP complex) or 2) mTORC2 heads to the ER independently of ribosomes and there it engages in mTORC2-ribosome interaction. Even though we observed mTOR-Srp14 interaction by masspectrometry in an earlier study (Adiel Cohen, unpublished), the results from a more detailed study of mTOR complex 2-SRP interaction were inconclusive (data not shown). We propose a model where mTORC2 goes to the ER/MAM and there engages in ribosome interaction.

2.2.1.4 *Material and Methods*

RIBOSOME PURIFICATION This protocol needs to be performed on ice or at 4 degrees celsius in absence of any RNases. Use $10-30$ 80% confluent 15cm plates of cells. For stimulations, cells were serum starved for 16h, pretreated with wortmannin for 5min (100nM) and stimulated with insulin 100nM $5-10$min. Wash 2x cold PBS, trypsinize, collect, spin 2500g 5m, add 20ml PBS cycloheximide (CHX, 100µg/ml) for 5m, count cells, spin 800g 5m, add 100µl buffer A per 10^7 cells, mix by pipetting 5 times p1000, wait 20m on ice, dounce until 90% lysis, pellet 8000g 5m, measure protein concentration. Layer $500-600$µl on top of 11ml 30% sucrose cushion, run at 39k g 17h. Invert tubes to empty sucrose, wipe with kimwipe. Pellets should be visible as clear glassy material attached to the bottom. Add $300-500$µl of buffer B, resuspend carefully, dounce 5x, measure protein concentration.

Buffer A: 100mM KCl, 5mM $MgCl_2$, 100mM Hepes, 0.3% CHAPS, 1mM DTT, 100U/ml RNAsin, Roche Complete Protease Inhibitors

Buffer B: 50mM Tris pH 7.5, 150mM NaCl, 2mM $MgCl_2$, 1mM DTT, 0.3% CHAPS, 100U/ml RNAsin, Roche Complete Protease Inhibitors

IMMUNOPRECIPITATION FROM RIBOSOMAL PELLETS To ribosomal lysate, add 4µg antibody, incubate with mixing end over end at 4 degrees 3h. Add 50µl of blocked microspheres in buffer B 1h. Wash 4x buffer B. Elute by adding 1ml RLT buffer, follow RNeasy Micro kit (QIAgen) instructions for purification of RNA, elute with 14µl water, check concentration by nanodrop, freeze at -80 degrees or use immediately.

Microspheres: Use 1µm Protein-A coated microspheres (Bangs Labs) following the manufactures' guidelines, pellet 10000g 2m, wash 2x, resuspend in buffer B plus 5% BSA plus 10µg/ml yeast tRNAs.

MICROARRAY ANALYSIS Microarray analysis was performed after quantification of mRNA and quality check in the Life Sciences Training Facility by Philippe Demougin on Affymetrix Human Gene 1.0 Array.

CLUSTER ANALYSIS The following settings were using while doing the cluster analysis on the mTORC2 enriched mRNAs: DAVID http://david.abcc.ncifcrf.gov. Background List HuGene-1.0-st-v1. Classification Stringency Medium. Kappa similarity: Similarity Term Overlap 3, Similarity Threshold 0.5. Classification: Initial Group Membership 3, Final Group Membership 3, Multiple Linkage Threshold 0.5.

2.2.2 *MAM integrity is important for mTORC2*

2.2.2.1 *Introduction*

Since ribosome association is important for mTORC2 activation and since mTORC2 is catalytically active at the ER and MAM, one obvious question is whether MAM localization is itself important for mTORC2 activity. One can imagine two possible scenarios where MAM integrity could be upstream of mTORC2: mTORC2 kinase activity might be affected by MAM, or mTORC2-substrate exhibition might depend on MAM, both leading to a similar outcome.

A possible hint that mTORC2 activity might indeed be affected under conditions where MAM integrity is reduced comes from the observation that the liver-specific knockout of Mfn2 leads to very similar phenotypes as liver specific Rictor deletion (as mentioned above) (Sebastián et al., 2012). Mfn2 knockout leads to a reduction of Akt S473 phosphorylation, indicating reduced mTORC2 activity. The authors concluded that elevated ER stress in these mice might inhibit insulin signaling.

2.2.2.2 *Results*

To see whether MAM formation is important for mTORC2 activity, we analyzed mTORC2 function in Mfn1/Mfn2 double knockout MEFs (Mfn dKO) that have a strong reduction in MAM formation (de Brito and Scorrano, 2008). The mitofusin proteins are important for mitochondrial fusion and form a molecular tether between the ER and the mitochondria in order to maintain MAM function (de Brito and Scorrano, 2008).

We find that in Mfn dKO cells, Akt S473 phosphorylation is severely reduced compared to control MEFs, while mTORC1 or PI3K activity is not affected (Figure 20A, page 68 and Figure 21A, page 69). This was confirmed by Mfn2 or Grp75 knockdown (Figure 20D, page 68). A similar observation on Akt S473 was recently reported in a liver-specific Mfn2 knockout mouse and reported to be due to an increase in ER stress (Sebastián et al., 2012), however we observe no change in ER stress levels in the Mfn dKO MEFs (Figure 21B, page 69). mTORC2 complex integrity is not affected in the Mfn dKO (Figure 20B-C, page 68). Concomitant with the defect in MAM formation in the Mfn dKO cells is the reduction of mTORC2 levels in the crude mitochondrial extract but not in purified ER of Mfn dKO cells (Figure 20F, page 68 and Figure 21C, page 69). Furthermore, we treated cells with ionomycin that leads to ER fragmentation and MAM disruption (Park et al., 2000; Goetz et al., 2007) and find a reduction in mTORC2 but not mTORC1 activity (Figure 20D, page 68).

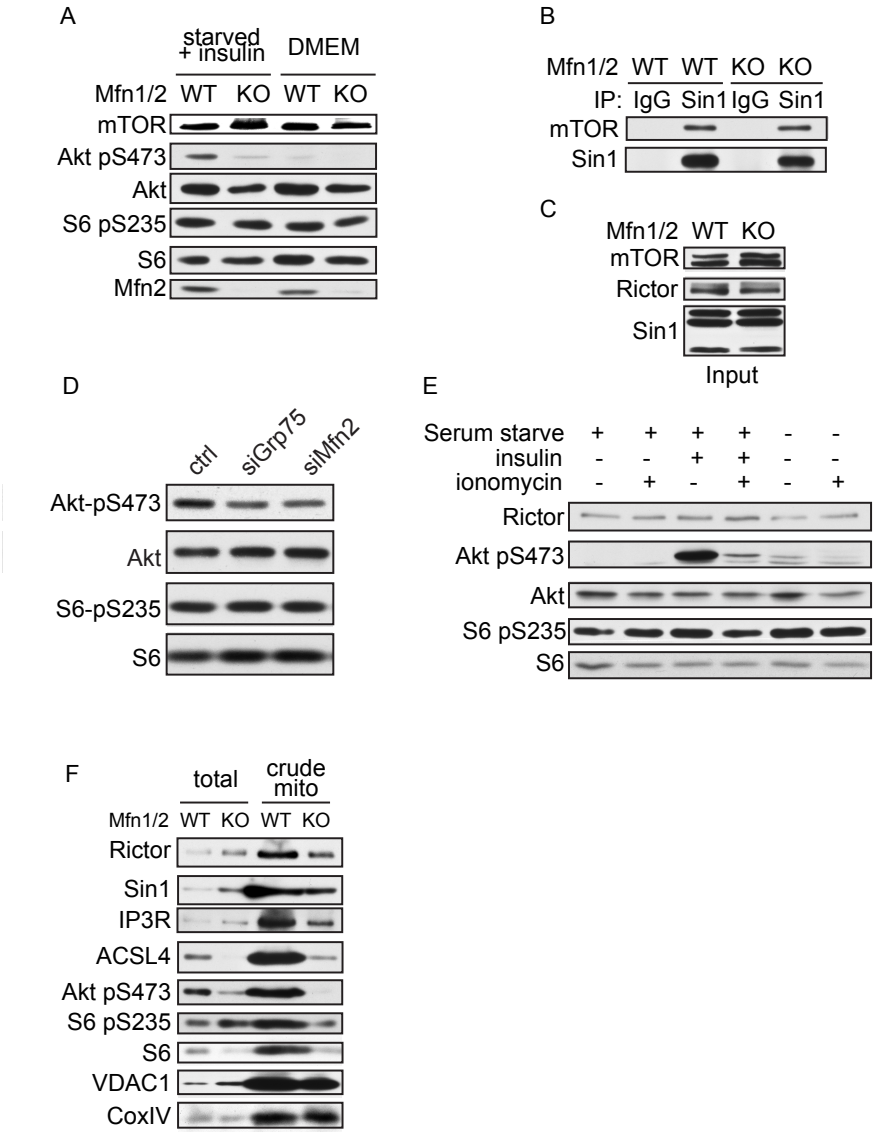

Figure 20: **mTORC2 activity depends on intact MAM. (A)** Insulin stimulated Akt-pS473 is reduced in Mfn1/2 dKO MEFs. **(B)** mTORC2 integrity is unchanged in Mfn dKO MEFs. **(C)** mTORC2 components are unchanged in Mfn dKO MEFs. **(D)** mTORC2 activity is reduced upon knockdown of Grp75 or Mfn2. **(E)** Ionomycin treatment, disrupting ER/MAM integrity, inhibits mTORC2 but not mTORC1 activity. **(F)** Mfn dKO MEFs show a reduction of MAM and mTORC2 components in a crude mitochondrial fraction composed of MAM and mitochondria.

2.2.2.3 *Discussion and outlook*

We observe that mTORC2 is both upstream and downstream of MAM. Previous results from a whole genome shRNA screen already showed the importance of mitochondrial function for mTORC2 addicted can-

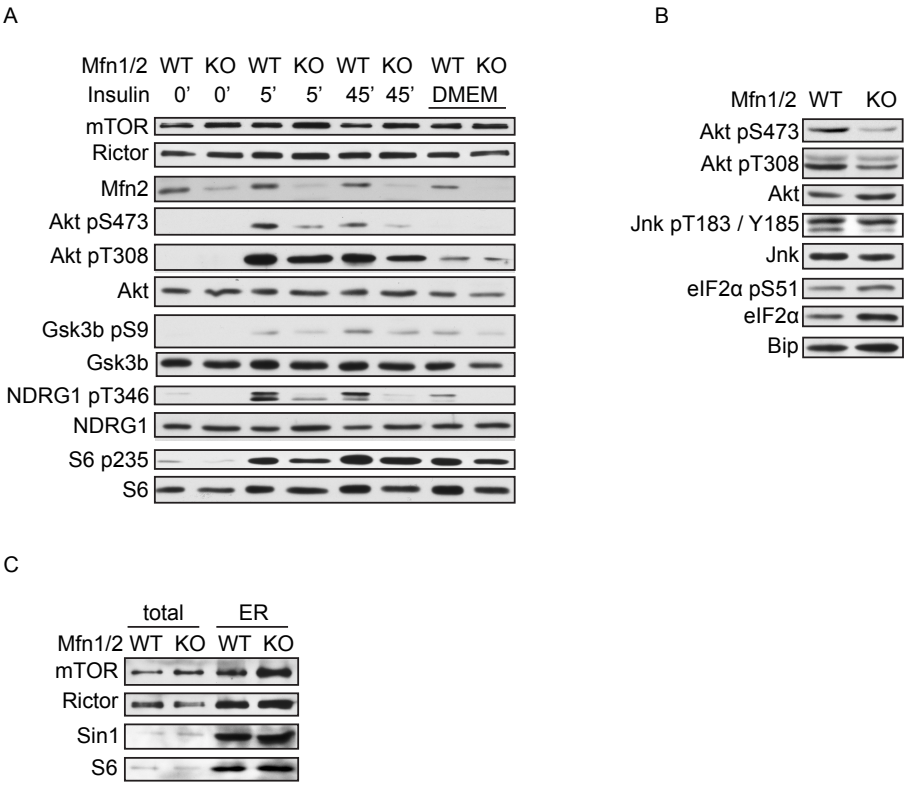

Figure 21: **Supplementary: mTORC2 activity depends on intact MAM. (A)** Time course of insulin stimulation in Mfn dKO MEFs shows reduced and not delayed mTORC2 activation. **(B)** Mfn dKO MEFs show no induction of ER stress response. **(C)** Mfn dKO MEFs show no reduction of mTORC2 components at the ER, indicating that mTORC2 can associate to the ER even if MAM is disrupted.

cer cells (Colombi et al., 2011). It may seem counterintuitive that one process is both upstream and downstream of a protein. However, the TOR signaling network presents multiple feedback loops (Efeyan and Sabatini, 2010) and many processes are both upstream and downstream of either one of the two TOR complexes, e.g. sphingolipid metabolism (Roelants et al., 2011; Tabuchi et al., 2006; Aronova et al., 2008), autophagy (Yu et al., 2010; Jung et al., 2010), amino acid signaling (Schmidt et al., 1998; Avruch et al., 2009) and ER stress (Tabas and Ron, 2011; Kato et al., 2012).

The molecular logic behind this additional loop might be a protection mechanism for the cell to endure starvation. Reduced mTORC2 signaling under these circumstances could cause a decrease in MAM in order to protect cells from apoptotic calcium fluxes from the ER while increasing the mitochondrial potential to stabilize the cellular energy levels, keeping the cells "locked" in a pre-apoptotic and anti-tumorigenic state. By the same logic, prolonged mTORC2 inactivity or disruption might cross this threshold and induce apoptosis, thus

presenting mTORC2 with the role of a buffer between apoptosis induction and execution. It will be interesting to see if under chronic mTORC2 activation, observed in many cancers, there is an increase in MAM formation and function, switching the cellular energy production from mitochondrial respiration to glycolysis.

We also show that mTORC2 activity is dependent on intact MAM and ER. This on the other hand might reflect a new sensory input to inform mTORC2 about the apoptotic stimuli that are transferred to the mitochondria via MAM.

We used Mfn1/2 double knockout MEFs. One might argue that Mfn2 (the predominant MAM tether) single knockout MEFs should be used instead. We tried to replicate our findings by knocking down Mfn2 with siRNA and could reduce Akt S473 phosphorylation (Figure 20D, page 68). The single knockout MEFs have been described, but are not commercially available in Europe. We were unsuccessful in obtaining these cells from the lab that originally generated them (Chen et al., 2005).

An interesting observation comes from the fact that even though there is reduced mTORC2 at MAM in the Mfn2 knockout cells (because of reduced MAM formation), mTORC2 levels at the ER are unaffected (Figure 20F, page 68 and Figure 21C, page 69). This indicates that mTORC2-MAM and not ER localization is important for its activity, thus underlining that mTORC2-MAM localization is not only a consequence of its ER localization, but that mTORC2-MAM association plays a functional role.

As discussed above, several pools of mTORC2 might exist. A global reduction of mTORC2 activity upon MAM disruption suggests that MAM localized mTORC2 might be predominant in this particular cell type or for the subset of mTORC2 targets observed. Alternatively, MAM localized mTORC2 might be mostly responsible for short-term, insulin mediated signaling, while other pools of mTORC2 might be more important for maintaining basal mTORC2 activity.

Liver-specific knockout mice for Mfn2 and mTORC2 share many phenotypes such as reduced Akt signaling, glucose intolerance and increased gluconeogenesis (Sebastián et al., 2012; Hagiwara et al., 2012). It is difficult to distinguish if this phenotype in the Mfn2 knockout mice is due to reduced mTORC2 activity or if on the other hand the phenotype in the Rictor knockout mice is due to reduced MAM formation. Cyclic pathways are inherently difficult to delineate, however one could imagine trying to rescue the phenotype of the Mfn2 knockout mouse by injecting it with adenoviral Akt S473D, thereby mimicking mTORC2 activity. The opposite approach, that is trying to restore MAM formation in the mTORC2 knockout liver, is more difficult, as it has been shown that overexpression of MAM tethers can lead to a disruption in MAM function.

The phenotypes of the liver-specific mTORC2 knockout mice were described to be due to a deficiency in glucokinase and SREBP1c activity (Hagiwara et al., 2012). We show in our study that hexokinase 2 at MAM is regulated by mTORC2 via Akt. Glucokinase (HK4) is closely related to HK2 and might be regulated in a similar manner. Indeed GK and hexokinase localize to MAM (Zhang et al., 2011). It is not exactly known how mTORC2 regulates SREBP1 maturation. Ceramides play a role in SREBP1c activation and we found that ceramide levels are reduced in mTORC2 knockout hepatocytes (Figure 25A, page 77).

mTORC2 activity is disrupted in Mfn1/2 dKO MEFs. However, we did not answer whether this is due to reduced kinase activity of mTORC2 or whether it is a lack of substrate availability that causes this defect, since Akt also localizes to MAM (Giorgi et al., 2010). A study from the Pandolfi laboratory suggested that Akt at MAM can be inhibited through the action of PP2a and PML (Giorgi et al., 2010). Considering this, it is surprising to see that Akt phosphorylation upon MAM disruption is decreased and not increased. We performed some preliminary mTORC2 kinase assays in the Mfn deficient MEFs and saw no change in mTORC2 activity toward a recombinant Akt substrate (data not shown). This suggests that mTORC2 substrate availability might be the cause for the decreased Akt phosphorylation in the Mfn dKO MEFs. However, as we immunoprecipitated mTORC2 from whole cell lysates for this assay, we cannot exclude that other potential pools of mTORC2 which might not depend on MAM integrity compensated for this loss of MAM. For future experiments, mTORC2 should be isolated from the MAM fraction to measure its activity upon Mfn deletion.

Phosphatidylserine (PS) can modulate mTORC2 activity toward Akt (Huang et al., 2011). We see that in the mTORC2 knockout livers, PS levels are reduced (Figure 25A, page 77). Since PS is synthesized at MAM, this might present one potential mechanism by which reduced MAM affects mTORC2 activity.

An interesting approach to further explore mTORC2 activity at MAM would be to target mTORC2 specifically to the ER or to MAM in order to test if this affects its activity. We tried targeting mTORC2 to the ER by a so called TA anchor, a tail that anchors proteins in a posttranslational manner to the cytosolic side of the ER. We introduced this tail into both Rictor and different Sin1 isoforms. However, this was unsuccessful, possibly because introduction of the tags prevented these artificial mTORC2 components from integrating into the complex (data not shown). It is inherently difficult to introduce tags into mTOR complex components, since they commonly lead to the exclusion of that component. In further experiments, we generated fusion proteins between mTORC2 and MAM or ER marker proteins. The outcome of these experiments was not successful either (data

not shown). A third approach of manipulating MAM targeting of mTORC2 by palmitoylation will be discussed below.

2.2.2.4 *Material and Methods*

CELL CULTURE Mfn1/2 dKO MEFs were purchased from ATCC and grown in DMEM, supplemented with 30% FCS, 2mM glutamine, 1X penicillin-streptomycin, 50µg/ml uridine. Cells proliferate slowly compared to their WT control MEFs and should be kept around $10-50\%$ confluency.

TREATMENT Cells were serum starved for 4h and stimulated with insulin 100nM for 10min. Ionomycin treatment was 0.5µM for 10min in HeLa cells.

KNOCKDOWNS siRNA smartpools were purchased from Dharmacon. We used 100nM siRNA per 6 well using Fugene HD (Roche) transfection reagent and protocol. Protein levels were assessed after 3 days.

2.2.3 *Differential localization of Sin1 isoforms*

2.2.3.1 *Introduction*

In order to reconcile previous reports of plasma membrane localization of mTORC2 (Saci et al., 2011) with our own observations, we hypothesized that there could be different pools of mTORC2. Three different mTORC2 complexes have been described previously (Frias et al., 2006). These complexes are defined by 3 Sin1 isoforms. Since mTORC2 might form dimers, this leads to a diversity of potential combinations. No functional differences are known between the Sin1 isoforms *in vivo*. Sin1.5 (the shortest isoform) lacks the Ras binding and PH domain and its activity does not respond to insulin *in vitro*. Sin1.2 lacks the Ras binding domain. The exact function of the Ras binding domain is not known, as previous studies were performed on tagged Sin1 isoforms that could not integrate into mTORC2 (Schroder et al., 2007).

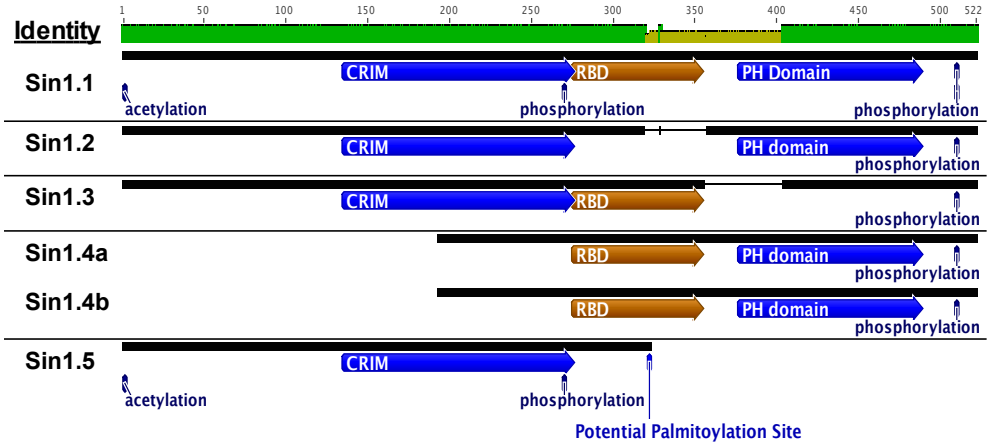

Figure 22: **Sin1 isoforms and modifications**. Alignment of the 5 different Sin1 isoforms and their respective domains.

2.2.3.2 *Results*

It had been previously observed that a shorter isoform of Sin1 was enriched in ribosomal pellets (Vittoria Zinzalla, unpublished results). We could confirm this observation, especially when comparing cytoplasmic extracts to ER extracts (Figure 23, page 74) and MAM (data not shown).

We had difficulties in identifying the exact Sin1 isoform that is enriched at the ER and MAM. We overexpressed different tagged Sin1 isoforms that had been previously published, but they were either not expressed in our hands (Frias et al., 2006) or were migrating at a different molecular weight than predicted (data not shown). What is clear though is that it is one of the shorter isoforms that is en-

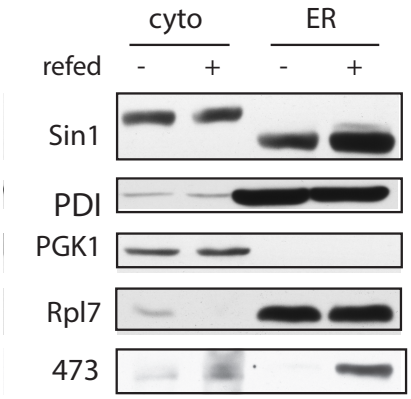

Figure 23: **Sin1 isoforms.** Sin1 isoforms in different subcellular lysates.

riched at the ER and MAM, thus limiting the possible isoforms to Sin1.2 and Sin1.5. Alternatively, the isoform might be differentially post-translationally modified at different organelles, thus changing its migration pattern.

We were interested as to what could be the reason for this differential localization, especially in the context of MAM localization. The only signal that is known to target a protein preferentially to MAM is palmitoylation. This post-translational modification targets proteins such as TMX and calnexin to MAM in a regulated manner (Lynes et al., 2011). 2-bromopalmitate, an inhibitor of palmitoylation inhibits Akt S473 phosphorylation (Watson et al., 2011).

	Position	Peptide	Score	Cutoff
Sin1.1	103	ERQNQIKCKNIQWKE	0.324	0.196
	231	NDNVSAYCLHIAEDD	0.778	0.408
	440	DSDLLCACDLAEEKS	0.889	0.408
Sin1.2	103	ERQNQIKCKNIQWKE	0.324	0.196
	231	NDNVSAYCLHIAEDD	0.778	0.408
	404	DSDLLCACDLAEEKS	0.889	0.408
Sin1.5	103	ERQNQIKCKNIQWKE	0.324	0.196
	231	NDNVSAYCLHIAEDD	0.778	0.408
	322	SQKVSGACD******	3.083	0.951

Figure 24: **Predicted palmitoylation of Sin1 isoforms.** Sin1 isoforms were analyzed for potential palmitoylation sites by the online application CSSPalm 3.0.

We analyzed the different Sin1 isoforms and mTORC2 components with a palmitoylation prediction algorithm termed CSSPalm 3.0 (http://csspalm.biocuckoo.org/online3.php). There were no significant hits discovered in any mTORC2 subunits except for Sin1.5, which

had a high of 3.083 (in the same range as positive control sites) (Figure 24, page 74). Scores under 1 are generally more difficult to interpret. This predicted palmitoylation needs to be confirmed experimentally in the future.

2.2.3.3 *Discussion and outlook*

We show preliminary data that there might be an enrichment of certain Sin1 isoforms at the ER. We have not included these observations in the main manuscript or developed them further because many necessary tools are still missing. We lack reliable Sin1 antibodies and functional Sin1 isoform specific expression vectors. The study of different pools of mTORC2 definitely deserves a thorough analysis that exceeds the scope of this project.

We tried re-expressing different Sin1 isoforms in Sin1 knockout MEFs generously provided by Estella Jacinto. However, these MEFs were not viable in our hands for more than 2-3 passages regardless of Sin1 transfection, thus limiting any further study. The design of Sin1 isoform specific siRNAs or antibodies is theoretically possible and might be of help in future studies.

The non-expressing Sin1 isoform plasmids obtained from Addgene (Frias et al., 2006) were resequenced in order to determine the cause for the lack of expression. No mutations in the coding sequences were found. The isoforms will be subcloned in a different expression vector in the future.

Specific tools for the identification of palmitoylation on a given site exist and include radioactive labeling with palmitate or non radioactive labels that require masspectrometry-based identification. These tools should be used in the future to confirm the possible palmitoylation of Sin1.

2.2.3.4 *Material and Methods*

SIN1 ISOFORMS Isoforms were visualized using Geneious software.

SIN1 PALMITOLYATION PREDICTION The online palmitoylation prediction server CSSPalm was used (`http://csspalm.biocuckoo.org/online3.php`). Threshold was set to medium stringency.

2.2.4 *mTORC2 regulates lipid trafficking at MAM*

2.2.4.1 *Introduction*

As stated before, the two main arms of MAM function are calcium and lipid trafficking between the ER and mitochondria. The calcium related MAM deficiencies in the mTORC2 knockout cells have been described earlier (Figure 13, page 50). Here we focus on the lipid compositions of different endomembranes in the mTORC2 knockout hepatocytes. As published previously, liver-specific Rictor knockout mice are hypolipidemic (Hagiwara et al., 2012). Here we isolated different subcellular organelles and purified their respective phospholipids, which were then subjected to MS analysis or detection by TLC.

2.2.4.2 *Results*

The following lipids were detected and quantified by masspectrometry: SM (sphingomyelin), PC (phosphatidylcholine), PE (phosphatidylethanolamine), PI (phosphatidylinositol), PS (phosphatidylserine), PA (phosphatidic acid), PG (phosphatidylglycerol) and Cer (ceramides). Massspectrometry allows further subclassification of these phospholipids but for simplicity, we show here only the average of each phospholipid class. Results are filtered for lipid species that were at or below the detection limit or that were not present in one of the fractions (total, ER, MAM, Mitochondria). Ratios of the lipid concentration from the knockout divided by the wildtype extracts are shown. Extracts were pooled from 3 different mice.

The total extracts from liver-specific mTORC2 knockout mice show a striking deficiency for most phospholipids (Figure 25A, page 77). A ratio of 1 indicates that the total levels of this lipid are not changed in the extract from the knockout mouse.

Since we were primarily interested in the distribution and trafficking of phospholipids, we also compared the respective lipid levels from MAM and mitochondrial fractions (Figure 25B, page 77). A ratio of 1 indicates a similar amount of a lipid species in its respective organelle between knockout and wild type. A lower ratio indicates that more of this lipid is present in the wildtype fraction. We can see from these results that many of the phospholipids are depleted from Rictor knockout mitochondrial extracts and enriched in knockout MAM extracts. This suggests that MAM-mitochondrial trafficking is reduced in these cells. Our findings were confirmed using TLC separation of the abundant phospholipids PS and PC (Figure 25C, page 77).

Complete dataset in Excel format will be available online under `http://edoc.unibas.ch` .

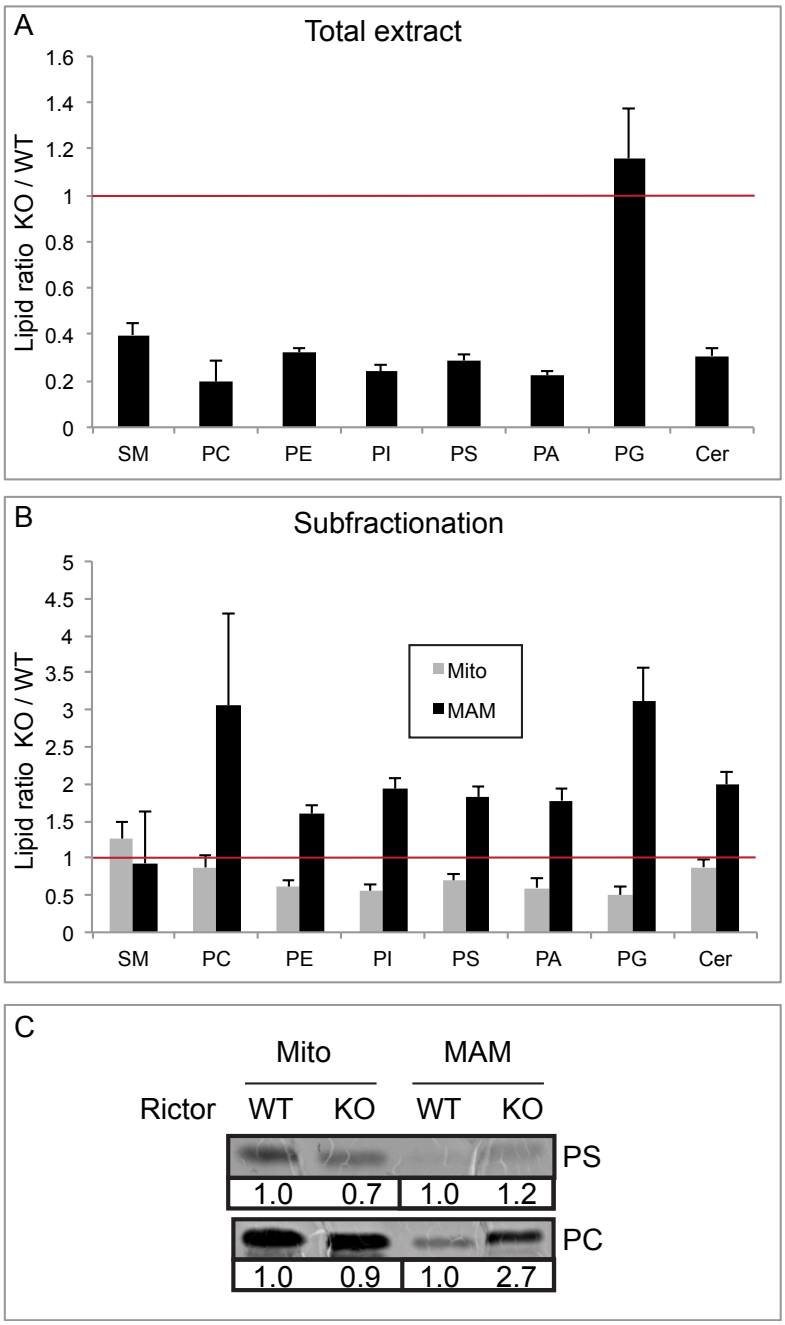

Figure 25: **Lipid synthesis and trafficking in Rictor knockout livers. (A)** Phospholipids isolated from total liver extracts of liver specific Rictor knockout mice relative to their controls. **(B)** Phospholipids isolated from MAM and mitochondrial extracts of liver specific Rictor knockout mice relative to their controls. Ratios > 1 indicate accumulation of the lipid in that specific organelle upon Rictor knockout. **(C)** PS and PC levels in MAM or mitochondrial fractions as resolved by TLC. Values show densitometric measurements of lipid intensities, normalized to wild type levels.

2.2.4.3 *Discussion and outlook*

In accordance with our previous results, we show that lipid trafficking between ER and mitochondria is deficient upon Rictor knockout. Furthermore, total phospholipid levels are reduced in the Rictor knockout cells, further underlining the importance of mTORC2 in lipid metabolism (Laplante and Sabatini, 2009b). It will be important for future research to distinguish whether the general defect in phospholipid synthesis in the mTORC2 knockout is caused directly by the defect in MAM. Evaluation of organelle lipid compositions from MAM deficient tissues (e.g. Mfn2 knockout livers) will be helpful in making this distinction.

These data are to our knowledge the first in depth study of MAM lipid composition. Unfortunately, very little information about MAM/mitochondrial lipid composition from other MAM deficient cells are available, so it is difficult to put our results into the context of a MAM deficiency.

As seen in Figure 5, page 21, the steps in phospholipid synthesis carried out at the ER side of MAM are PA to PS and PE to PC. PS to PE is carried out on the mitochondrial side of the MAM junction. We see an accumulation of all of these lipid species at MAM of mTORC2 deficient cells, while observing a decrease in their levels at mitochondria, respectively. We are uncertain if PE, also accumulated at MAM of Rictor knockout cells, is synthesized at mitochondria or at another site such as the ER.

The results from the MS analysis presented here should be considered as preliminary. Even though we pooled extracts from 3 different mice from each genotype, the entire detection should be repeated with more samples in the future. Furthermore, more lipids species should be analyzed independently by TLC separation.

We present the measurements as the average levels for each class of lipid species. For example, 48 different subspecies of PS can be detected (PS(31:1)-PS(44:2)). Furthermore, we filtered out low abundant species that represented only minorities of their respective class. A more detailed evaluation of the different subspecies of these lipid classes might provide new insights into their function in the context of mTORC2 or MAM.

2.2.4.4 *Material and Methods*

ORGANELLE ISOLATION Purification of different organelles was performed as previously described(Wieckowski et al., 2009).

PHOSPHOLIPID EXTRACTION Protocol was performed as required by the lipidomics facility (`http://www.k-state.edu/lipid/lipidomics/index.htm`)

Use glass tubes with Teflon-lined screw caps. To 0.8 parts cells/tissue (homogenized) in aqueous solution, add 1 part chloroform and 2 parts methanol. Shake well, add 1 part chloroform and 1 part water. Shake, centrifuge at low speed for 5-10 min. Remove the lower layer. Add 1 part chloroform, shake, centrifuge, remove the lower layer. Add 1 part chloroform, shake, centrifuge, remove the lower layer. Wash the combined lower layers once with a small volume 1 M KCl and once with a small volume of water. Fill tubes with nitrogen, store in freezer; evaporate the solvent, fill the tubes with nitrogen, and ship on dry ice. If you have trouble with emulsions, you may have to add some KCl or other salt to the extraction.

PHOSPHOLIPID ANALYSIS Analysis was performed by the Kansas Lipidomics Research Center (KLRC) at Kansas State University. Samples were normalized to dry lipid weight.

2.2.5 *mTORC2 controls body temperature*

2.2.5.1 *Introduction*

We were interested in the consequences of the MAM defects caused by mTORC2 disruption on whole body physiology. Unfortunately, very little work has been done toward generating tissue-specific knockout mice for MAM proteins such as Grp75, PACS2 or IP3R3. It is likely that there are differences in the importance of MAM function between tissues such as the liver, the muscle and particularly the brain because of neurological disorders.

To test if mTORC2-MAM regulation is a general phenomenon not restricted to the liver and or cultured cells, we asked whether mTORC2 knockout in the adipose tissue also leads to MAM related phenotypes. MAM in white adipose tissue supposedly plays only a minor role compared to the liver. The Ad-Cre driver used to generate the adipose-specific mTORC2 knockout mice however is also expressed in the brown adipose tissue. The ER and mitochondria in this specific organelle play a very central role in thermogenesis. MAM in the brown adipose tissue has been linked to the regulation of thermogenesis (Kuba et al., 2007; de Meis et al., 2010).

We asked therefore if the core body temperature is affected in the adipose-specific Rictor knockout mice; a phenotype previously uncharacterized in these mice (Cybulski et al., 2009).

2.2.5.2 *Results*

The mice were 37 weeks of age, on a standard chow diet and under anesthesia for 5 minutes before the core body temperature was measured. As can be seen in Figure 26, page 80, adipose-specific Rictor knockout mice (AdRicKO) displayed an average reduction of 1.5°C in their core body temperature when compared to their wild type littermate controls (AdRicWT) (3 mice per group).

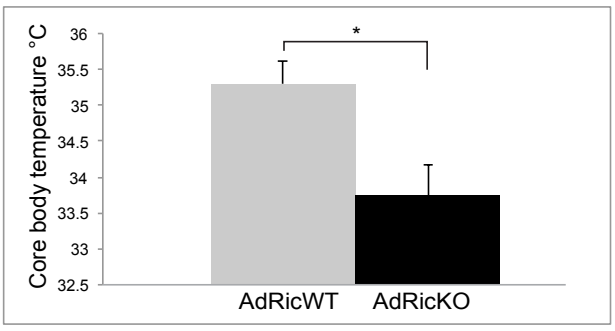

Figure 26: **Body temperature in AdRicKO mice versus wildtype littermates**. AdRicKO mice show an average drop of 1.5 degrees Celsius in their core body temperature.

2.2.5.3 *Discussion and Outlook*

We observe a reduction in the body temperature of AdRicKO mice. We wanted to correlate these findings with a reduction in MAM formation that we would expect upon mTORC2 knockout in this tissue. However, due to the small size of the brown adipose tissue, analysis of MAM integrity by biochemical fractionation is unfeasable. Instead we tried to evaluate any potential MAM deficiencies by electron microscopy. Unfortunately, we failed to establish a protocol that preserved the integrity of MAM during embedding. Together with our collaborator Cristina Baschong, we will try to further optimize this protocol in the future. Alternatively, we could measure the mitochondrial uncoupling of this tissue in order to see if the the reduced body temperature is caused by the defect in the brown adipose tissue (BAT) uncoupling that might be due to a MAM deficiency. Of note, no difference in the amount of BAT was detected between wild type and Rictor knockout (Cybulski et al., 2009). The AdRicKO mice have an increase in body size, however, there is no correlation between body size and body temperature in mice (Rhodes et al., 2000).

The measured difference in the body temperature is substantial. Genetic ablation of BAT in mice leads to an average reduction in body temperature of about 1°C (Klaus et al., 1998).

Future studies involving these mice are limited by the fact that due to space limitations, this breeding has been reduced to a minimum in house. Our collaborators in Zurich have expressed interest in pursuing this study further in the future.

2.2.5.4 *Material and Methods*

MEASUREMENTS These results were generated with the help of our collaborator Dr. Elvira Haas from the University Hospital of Zurich.

3

APPENDIX

3.1 LIVER-SPECIFIC KNOCKOUT OF RICTOR

During my PhD thesis, I established the technique of hepatocyte isolations in our lab. I was involved in the characterization of the liver-specific knockout of mTORC2 by isolating primary hepatocytes and by generating and analyzing the immunofluorescent images. The main characterization of these mice was performed by Asami Hagiwara. As many of the lysates used in my study come from liver-specific Rictor knockout mice and their controls, this study is highly relevant to my work and presented on the following pages.

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Cell Metabolism
Article

Hepatic mTORC2 Activates Glycolysis and Lipogenesis through Akt, Glucokinase, and SREBP1c

Asami Hagiwara,[1] Marion Cornu,[1,4] Nadine Cybulski,[1,4] Pazit Polak,[1] Charles Betz,[1] Francesca Trapani,[2] Luigi Terracciano,[2] Markus H. Heim,[3] Markus A. Rüegg,[1] and Michael N. Hall[1,*]
[1]Biozentrum, University of Basel, CH-4056 Basel, Switzerland
[2]Institute of Pathology
[3]Department of Biomedicine
University Hospital, CH-4003 Basel, Switzerland
[4]These authors contributed equally to this work
*Correspondence: m.hall@unibas.ch
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SUMMARY

Mammalian target of rapamycin complex 2 (mTORC2) phosphorylates and activates AGC kinase family members, including Akt, SGK1, and PKC, in response to insulin/IGF1. The liver is a key organ in insulin-mediated regulation of metabolism. To assess the role of hepatic mTORC2, we generated liver-specific *rictor* knockout (LiRiKO) mice. Fed LiRiKO mice displayed loss of Akt Ser473 phosphorylation and reduced glucokinase and SREBP1c activity in the liver, leading to constitutive gluconeogenesis, and impaired glycolysis and lipogenesis, suggesting that the mTORC2-deficient liver is unable to sense satiety. These liver-specific defects resulted in systemic hyperglycemia, hyperinsulinemia, and hypolipidemia. Expression of constitutively active Akt2 in mTORC2-deficient hepatocytes restored both glucose flux and lipogenesis, whereas glucokinase overexpression rescued glucose flux but not lipogenesis. Thus, mTORC2 regulates hepatic glucose and lipid metabolism via insulin-induced Akt signaling to control whole-body metabolic homeostasis. These findings have implications for emerging drug therapies that target mTORC2.

INTRODUCTION

Target of rapamycin (TOR) is a highly conserved protein kinase that controls cell growth and metabolism in response to nutrients, growth factors, and energy status. TOR exists in two structurally and functionally distinct complexes termed TOR complex 1 (TORC1) and TORC2 (Loewith et al., 2002). Mammalian TORC1 (mTORC1) contains mTOR, raptor, and mLST8 and phosphorylates a variety of substrates to control protein synthesis, ribosome biogenesis, autophagy, and other growth-related processes (Laplante and Sabatini, 2009; Polak and Hall, 2009; Russell et al., 2011; Wullschleger et al., 2006). The two best-characterized mTORC1 substrates are ribosomal protein S6 kinase (S6K) and eukaryotic initiation factor 4E-binding protein (4E-BP), both of which control protein synthesis. mTORC2 comprises mTOR, rictor, mSin1, mLST8, and PRR5 (also known as protor) and phosphorylates members of the AGC kinase family, including Akt (also known as PKB), SGK1, and PKC, via which mTORC2 controls cell survival, actin cytoskeleton organization, and other processes (Cybulski and Hall, 2009; García-Martínez and Alessi, 2008; Ikenoue et al., 2008; Jacinto et al., 2006; Jacinto et al., 2004; Sarbassov et al., 2004; Yang et al., 2006). By regulating a wide range of anabolic and catabolic processes, the mTOR complexes play a key role in growth, development, metabolism, and aging and are implicated in a variety of pathological states including cancer, obesity, and diabetes (Dazert and Hall, 2011).

Many studies with genetically modified animals indicate mTOR signaling plays a role in whole-animal metabolism and in the development of disease. Full-body knockout of any component of mTORC1 or mTORC2 causes embryonic lethality (Gangloff et al., 2004; Guertin et al., 2006; Jacinto et al., 2006; Murakami et al., 2004; Shiota et al., 2006; Yang et al., 2006). More recent studies have focused on mTOR function specifically in metabolic tissues, in large part due to these tissues being particularly sensitive to the three inputs that control mTOR (nutrients, insulin, and energy status). Conditional knockout of *raptor* (mTORC1) in skeletal muscle results in muscle dystrophy, glucose intolerance, and short lifespan, whereas knockout of *rictor* (mTORC2) in skeletal muscle confers little to no phenotype (Bentzinger et al., 2008; Kumar et al., 2008). Adipose-specific *raptor* knockout mice display increased energy expenditure and resistance to diet-induced obesity (Polak et al., 2008). Adipose-specific *rictor* knockout mice are characterized by increased glucose metabolism and somatic growth due to high circulating levels of insulin and IGF1 (Cybulski et al., 2009; Kumar et al., 2010). In podocytes, mTORC1 plays a role in the development of diabetic nephropathy, whereas mTORC2 appears to have a minor role (Gödel et al., 2011; Inoki et al., 2011). Recent findings suggest that hepatic mTORC1 controls ketogenesis and possibly lipid metabolism (Kenerson et al., 2011; Sengupta et al., 2010; Yecies et al., 2011). Together, the above studies suggest that the two mTOR complexes contribute to whole-body metabolic homeostasis

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via distinct roles in different metabolic tissues. However, the role of mTORC2 in the liver remains to be determined.

The liver plays a central role in whole-body glucose and lipid homeostasis (Postic et al., 2004). In the fasted state, the liver maintains blood glucose levels by producing glucose via glycogen breakdown and via gluconeogenesis. In the postprandial state (i.e., satiety—the increased availability of glucose and insulin), the liver ceases to produce glucose and takes up excess circulating glucose to replenish glycogen and triglyceride (TG) stores. Insulin is the major hormone controlling the fasted to postprandial transition (Saltiel and Kahn, 2001). In type 2 diabetes, hepatic insulin resistance leads to altered glucose metabolism and thereby hyperglycemia. Insulin signals through the PI3K-Akt pathway to inhibit gluconeogenesis and activate glycolysis and lipogenesis. Akt inhibits expression of gluconeogenic genes, by inhibiting FoxO (Puigserver et al., 2003), and induces glycolytic and lipogenic genes, by activating sterol regulatory element-binding protein 1c (SREBP1c) and glucokinase (GK). SREBP1c is a transcription factor that promotes expression of a number of lipogenic genes (Horton et al., 2002). GK, the rate-limiting enzyme of glycolysis in the liver, stimulates glycolysis and lipogenesis by enhancing glucose flux, including production of acetyl-CoA for lipid synthesis (Foufelle and Ferré, 2002). Furthermore, GK stimulates glycolysis and lipogenesis at the transcriptional level via the carbohydrate responsive element-binding protein (ChREBP) (Uyeda and Repa, 2006). Thus, the combined action of insulin signaling and glucose flux regulates glucose and lipid metabolism in the liver.

mTORC2 phosphorylates Ser473 in the so-called hydrophobic motif of Akt and thereby activates Akt toward some but not all substrates (Guertin et al., 2006; Jacinto et al., 2006; Sarbassov et al., 2005; Shiota et al., 2006; Yang et al., 2006; Zinzalla et al., 2011). To elucidate the role of mTORC2 in the liver in vivo, we generated liver-specific *rictor* knockout mice and investigated Akt signaling and glucose and lipid metabolism. We find that the mTORC2-deficient liver is unable to sense the state of satiety. Hepatic mTORC2 activates both Akt signaling and glucose flux to control glucose and lipid metabolism in the liver and thereby overall metabolic homeostasis.

RESULTS

mTORC2 Deficiency in the Liver Leads to Hyperglycemia, Hyperinsulinemia, and Hypolipidemia

To investigate the role of mTORC2 in the liver, we generated mice lacking rictor, an essential and specific component of mTORC2, exclusively in the liver (see Experimental Procedures). *Li*ver-specific *ri*ctor *k*nockout (*rictor*$^{fl/fl}$ *Alb-Cre*$^{Tg/0}$) mice (LiRiKO mice) were viable, born at the expected frequency for Mendelian inheritance, and showed normal fertility. In all subsequent experiments, littermates without the *Cre* transgene (*rictor*$^{fl/fl}$) were used as controls. In LiRiKO mice, rictor protein was absent only in the liver (Figure S1A). To evaluate mTORC2 activity, we investigated the phosphorylation status of mTORC2 substrates in the liver of mice treated with insulin. mTORC2 phosphorylates the hydrophobic (Ser473) and turn (Thr450) motifs in Akt (Facchinetti et al., 2008; Ikenoue et al., 2008; Jacinto et al., 2006; Sarbassov et al., 2005; Yang et al., 2006). Akt phosphorylation at these two sites was significantly reduced in LiRiKO liver (Figure 1A). Phosphorylation of Akt Thr308, a site in the catalytic loop phosphorylated by PDK1, was normal. mTORC2 also phosphorylates and thereby controls the stability and activity of PKCα and SGK1, respectively (García-Martínez and Alessi, 2008; Ikenoue et al., 2008). As expected, PKCα protein levels and phosphorylation of the SGK1 substrate NDRG1 were decreased in LiRiKO liver (Figure 1A). These observations confirm that both rictor protein and mTORC2 activity are absent in the liver of LiRiKO mice.

We next examined systemic parameters of LiRiKO mice fed a normal (chow) or high-fat diet (HFD). On a chow diet, LiRiKO mice showed normal growth rates (Figure 1B) and normal body composition (Figure S1B). When fed a HFD for 10 weeks, LiRiKO mice were slightly lighter than controls, but this difference was not statistically significant (Figure S1C). After 20 weeks on a HFD, fat mass was significantly reduced (5%) in LiRiKO mice (Figure S1D). This was likely due to increased lipolysis and mitochondrial oxidation in adipose tissue of LiRiKO mice, as suggested by an increased level of plasma glycerol (Figure S1E) and increased expression of genes involved in lipolysis and mitochondrial oxidation in adipose tissue (Figure S1F). Analysis of plasma parameters revealed that plasma TG and cholesterol (total and HDL cholesterol) levels were significantly lower (Figure 1C), while plasma glucose (Figure 1D) and insulin (Figure 1E) levels were significantly higher, in both fasted and chow-fed LiRiKO mice. The same differences in blood parameters were observed with mice on a HFD (Figures S1G–S1I). The levels of ALT and AST were similar for the two genotypes (Figures S1J and S1K). Thus, deletion of mTORC2 in the liver leads to hyperglycemia, hyperinsulinemia, and hypolipidemia, suggesting that hepatic mTORC2 mediates metabolic homeostasis.

The liver of LiRiKO mice displayed normal gross morphology (data not shown), and normal cell size and histopathology (Figure 1F). However, the weight of the liver of LiRiKO mice was 20% less compared to controls, for mice fed ad libitum either a chow or a HFD (Figures 1G and S1L). This difference was not observed in fasted mice (Figure S1M). Further analysis revealed that glycogen and TG levels in the liver of fed LiRiKO mice were significantly lower compared to controls (Figures 1F, 1H, 1I, and S1N). Thus, loss of hepatic mTORC2 results in a smaller liver with reduced glycogen and TG content in fed mice.

Deletion of mTORC2 in the Liver Causes Glucose Intolerance

LiRiKO mice developed mild hyperglycemia and hyperinsulinemia at a young age (6 weeks), indicating that impaired glucose homeostasis is an early effect of mTORC2 loss in the liver (Figures S2A and S2B). To evaluate further whole-body glucose homeostasis, we performed a glucose tolerance test (GTT) and an insulin tolerance test (ITT). In the GTT, LiRiKO mice exhibited significantly higher blood glucose levels before and after glucose administration (Figure 2A). Plasma insulin levels were also significantly higher in LiRiKO mice (Figure 2B). The glucose intolerance became more pronounced in older (>8 months) LiRiKO mice (Figure S2C). In the ITT, when the mice were fasted for 6 hr before the experiment, LiRiKO mice showed normal insulin sensitivity (Figure 2C). In contrast, when the mice were fasted overnight, LiRiKO mice showed slightly but significantly decreased glucose clearance (Figure 2D). The fact that lower

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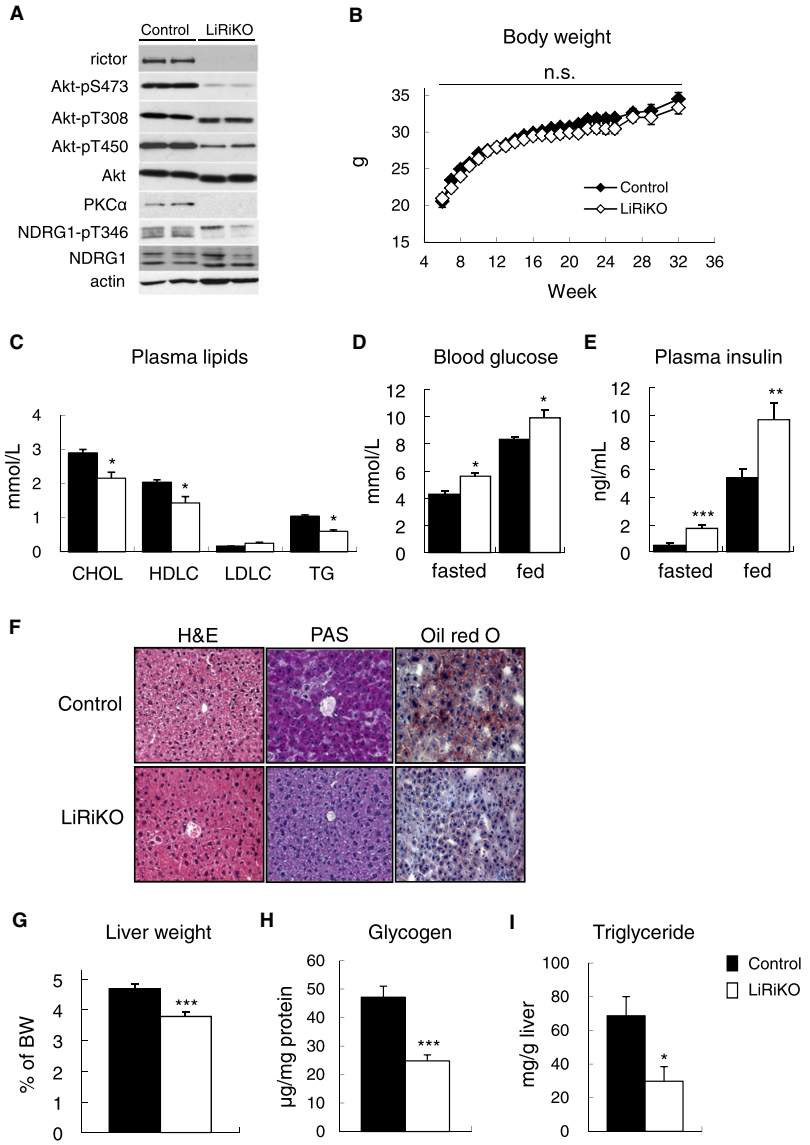

Figure 1. LiRiKO Mice Develop Hyperglycemia, Hyperinsulinemia, and Hypolipidemia

(A) Western blots showing loss of rictor expression and mTORC2 activity in the liver of LiRiKO mice. Fasted mice were injected with insulin as described in Experimental Procedures.

(B) Body weight (BW) gain of mice on a chow diet (n = 12 for control, n = 19 for LiRiKO). BW was monitored every week from 6 weeks of age for 26 weeks.

(C) Plasma total cholesterol (CHOL), HDL cholesterol (HDLC), LDL cholesterol (LDLC), and TG levels of ad libitum fed mice (n = 6 per group).

(D and E) Blood glucose (D) and plasma insulin (E) levels of overnight fasted and ad libitum fed mice (n = 8 for control and n = 6 for LiRiKO).

(F) Representative images of H&E, PAS, and oil red O staining of liver sections from ad libitum fed mice. Original magnification, ×40.

(G) Liver weight (normalized to BW) of 10- to 18-week-old mice fed ad libitum (n = 12–13 per group).

(H) Hepatic glycogen content of mice after 4 hr refeeding (n = 7 per group).

(I) Hepatic TG content of mice after 4 hr refeeding (n = 7 per group). Black bars represent control mice and white bars represent LiRiKO mice. Values are expressed as mean ± SEM. * indicates statistical significance from control mice (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

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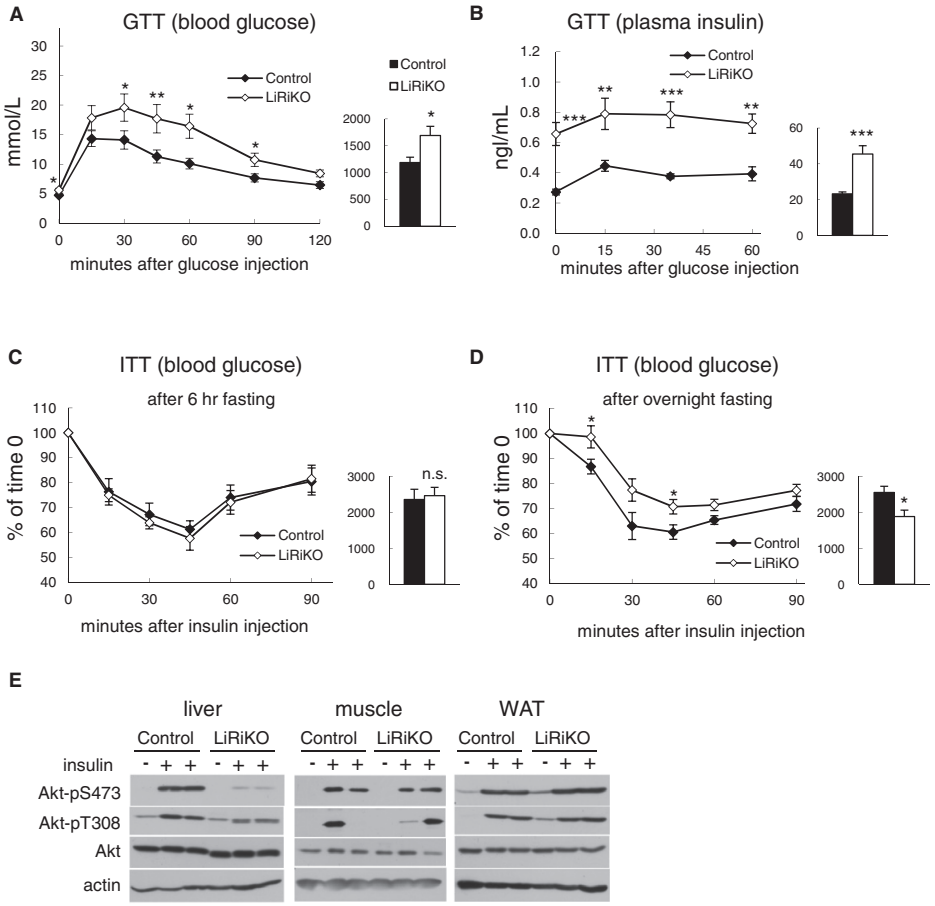

Figure 2. Deletion of mTORC2 in the Liver Causes Glucose Intolerance

(A and B) Glucose tolerance test (GTT) in overnight fasted 14-week-old mice. Mice were injected with glucose (2 g/kg) and blood glucose (A) and plasma insulin (B) levels were measured at the indicated times. Bar graphs to the right show the respective area under the curve (AUC) of glucose and insulin (n = 7–8 per group).

(C and D) Insulin tolerance test (ITT) in 6 hr fasted (C) and overnight fasted (D) 16-week-old mice. Mice were injected with insulin (0.5 or 0.25 IU/kg, ip), and blood glucose levels were measured at the indicated times. Results are expressed as percentage of the initial blood glucose levels. Bar graphs to the right show the respective inverse area under the curve (AUC) of glucose (n = 6–7 per group).

Values are expressed as mean ± SEM. * indicates statistical significance from control mice (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

(E) Insulin-stimulated phosphorylation of Akt in the liver, skeletal muscle, and epididymal WAT of control and LiRiKO mice. Fourteen-week-old mice were injected with insulin as described in Experimental Procedures.

insulin sensitivity in LiRiKO mice was observed only after overnight fasting, when hepatic gluconeogenesis is more active, suggests that LiRiKO mice have hepatic insulin resistance but normal insulin sensitivity in other tissues. Consistent with this, insulin-induced activation of Akt was normal in both skeletal muscle and adipose tissue in LiRiKO mice (Figure 2E). However, older LiRiKO mice (>8 months) showed impaired glucose clearance even after 6 hr fasting (Figure S2E), suggesting that whole-body insulin sensitivity decreased with age as a consequence of chronic hyperinsulinemia. When fed a HFD, LiRiKO mice displayed glucose intolerance (Figure S2F) and moderate insulin resistance (Figure S2G) already at 14 weeks of age. Thus, LiRiKO mice develop diabetes (glucose intolerance, hyperinsulinemia, and insulin resistance), further suggesting that hepatic mTORC2 controls whole-body metabolic homeostasis.

Hepatic mTORC2 Is Required for Insulin-Akt Signaling to FoxO1 and GSK3α/β but Not to mTORC1

The above findings suggest that the primary defect of a hepatic mTORC2 deficiency may be insulin resistance in the liver. To investigate this further, we examined insulin signaling, in particular insulin-activated Akt signaling, in the liver. Previous studies have shown that mTORC2 (i.e., Akt Ser473 phosphorylation) appears to be necessary for Akt activity toward some but not

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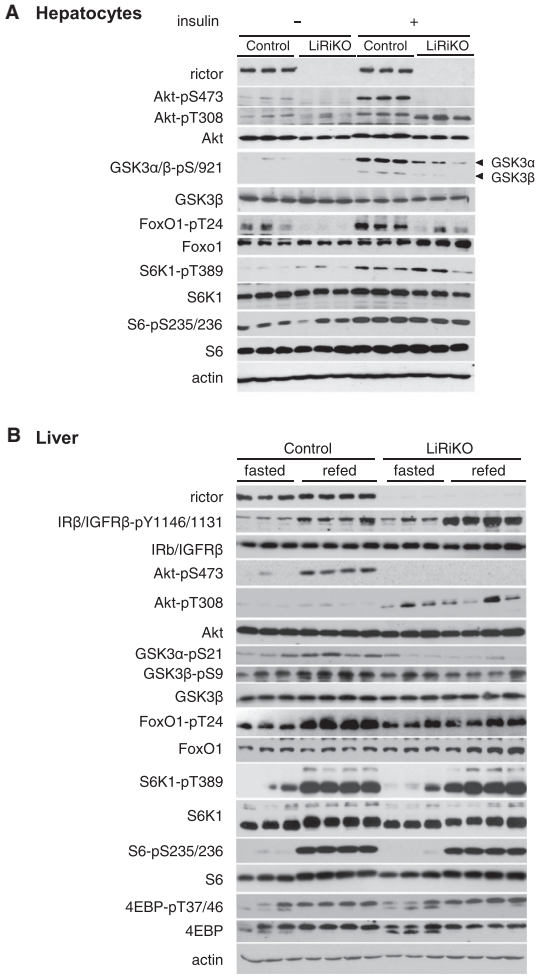

Figure 3. Hepatic mTORC2 Is Required for Insulin-Akt Signaling to FoxO1 and GSK3α/β but Not to mTORC1

(A) Western blots of signaling molecules involved in the Akt and mTORC1 pathway in primary hepatocytes stimulated with 50 nM insulin for 30 min.

(B) Western blots of signaling molecules involved in the Akt and mTORC1 pathway in the liver of fasted and refed mice. Fourteen- to sixteen-week-old mice (n = 4–6 per condition) were either fasted or refed a chow diet for 2 hr.

all substrates (Guertin et al., 2006; Jacinto et al., 2006; Polak and Hall, 2006; Sarbassov et al., 2005; Shiota et al., 2006; Yang et al., 2006). We first analyzed the phosphorylation status of Akt and several Akt downstream effectors in insulin-stimulated primary hepatocytes. In control hepatocytes, insulin stimulated phosphorylation of Akt (Ser473 and Thr308), GSK3α/β (Ser21 and Ser9), FoxO1 (Thr24), S6K (Thr389), and S6 (Ser235/236) (Figure 3A). In LiRiKO hepatocytes, insulin failed to stimulate Akt Ser473 phosphorylation whereas Thr308 phosphorylation was normal (Figure 3A), confirming our in vivo findings described above. The Akt substrates GSK3α/β and FoxO1 were significantly hypophosphorylated in insulin-treated LiRiKO hepatocytes (Figure 3A). Importantly, phosphorylation of the Akt downstream effectors S6K and S6 was normal, supporting the previous finding that Ser473 phosphorylation is not necessary for Akt to signal to mTORC1. Next, we examined Akt effectors in the liver of fasted and refed mice. Refeeding stimulated phosphorylation in control and LiRiKO livers in the same manner as observed in insulin-stimulated control and LiRiKO hepatocytes (Figure 3B). The only noteworthy exception in results obtained with hepatocytes versus livers is that Akt Thr308 was hyperphosphorylated in the liver of fasted and refed LiRiKO mice (Figure 3B). This is consistent with the hyperinsulinemia and increased tyrosine phosphorylation of the insulin receptor in LiRiKO mice (Figure 3B). Thus, hepatic mTORC2 is required for insulin-Akt signaling to FoxO1 and GSK3α/β, but not for insulin-Akt signaling to mTORC1. Furthermore, these findings confirm hepatic insulin resistance, albeit partial, in LiRiKO mice.

Loss of mTORC2 Results in Dysregulated Hepatic Gluconeogenesis and Glycolysis

How might the observed defect in hepatic insulin signaling lead to defects in metabolic homeostasis, e.g., hyperglycemia? As shown above, FoxO1 is hypophosphorylated in the liver of refed LiRiKO mice (Figure 3B). FoxO1 is a transcription factor that functions with the transcriptional coactivator PGC1α to induce gluconeogenic genes such as glucose-6-phosphatase (G6Pase) and phosphoenolpyruvate carboxykinase (PEPCK) in the fasted state. During the fasted to postprandial transition, FoxO1 is phosphorylated by Akt, which results in nuclear exclusion of FoxO1 and inihibition of gluconeogenesis (Matsumoto et al., 2007; Puigserver et al., 2003). Consistent with hypophosphorylation of FoxO1 in the liver of LiRiKO mice, nuclear exclusion of FoxO1 in response to feeding was severely impaired (Figure 4A). Furthermore, visualization of FoxO1 by immunostaining revealed that FoxO1 is preferentially localized in the nucleus in LiRiKO hepatocytes (Figures 4B and S3A). To further investigate the regulation of gluconeogenesis, we examined glucose production and gluconeogenic gene expression. LiRiKO hepatocytes exhibited higher basal glucose production, which was less inhibited by insulin, as compared to controls (Figure 4C). Consistent with these observations, mRNA levels of PEPCK and G6Pase in basal and insulin-treated conditions were significantly higher in LiRiKO hepatocytes (Figure S3B). Higher mRNA levels of gluconeogenesis-related genes were also observed in the liver of refed LiRiKO mice (Figure 4D). Finally, in a pyruvate challenge test, LiRiKO mice showed mildly but significantly higher blood glucose levels compared to controls, further suggesting increased hepatic glucose production capacity (Figure S3C). Thus, gluconeogenesis is constitutively active in the liver of LiRiKO mice, accounting for the observed high levels of blood glucose in both fasted and fed LiRiKO mice.

To further investigate the cause of the hyperglycemia, we examined hepatic glucose uptake and glycolysis. Glucose uptake was significantly reduced in LiRiKO hepatocytes compared to control hepatocytes (Figure 4E). Glucose uptake by hepatocytes is determined by the rate of glycolysis which is mainly dependent on GK activity (Ferre et al., 1996). GK, the first enzyme in the glycolytic pathway, is induced by insulin

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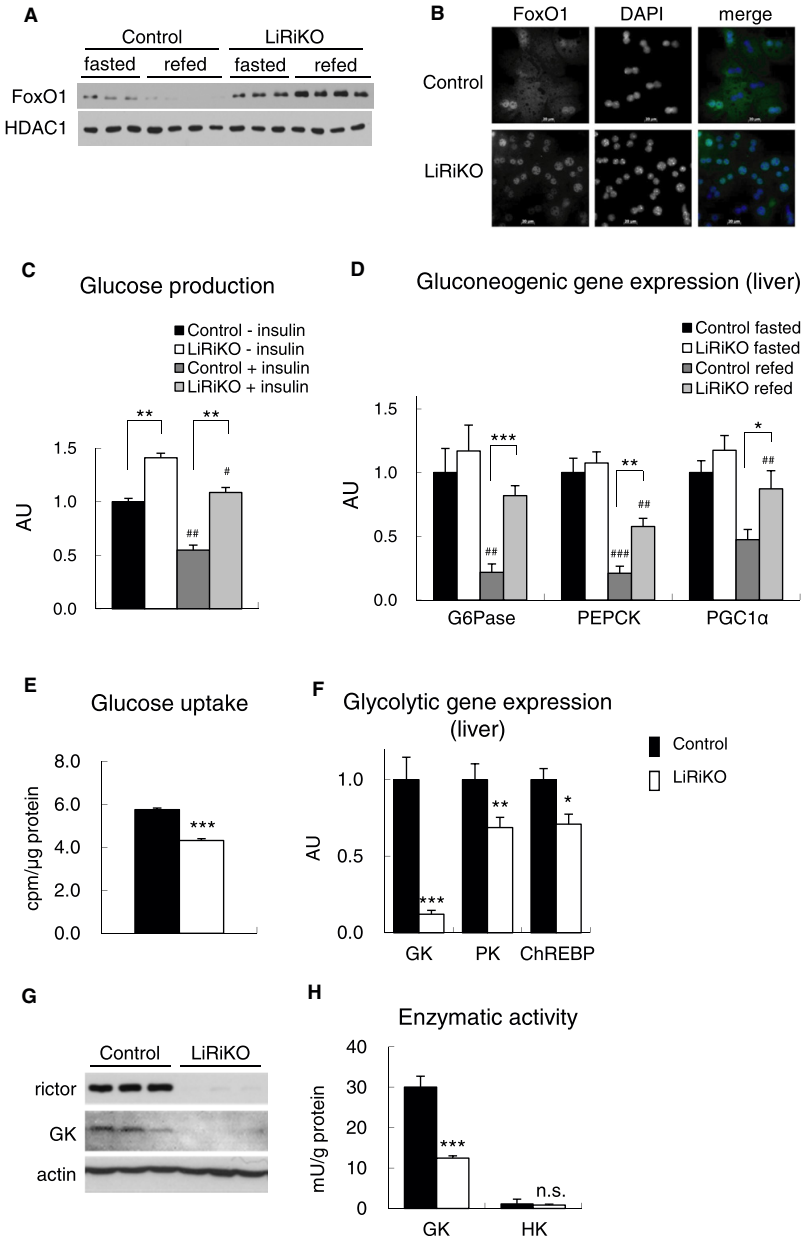

Figure 4. Loss of mTORC2 Results in Active FoxO1 and Constitutively Elevated Hepatic Gluconeogenesis, Reduced Glucokinase Expression, and Glucose Flux

(A) FoxO1 protein levels in nuclear fractions from the liver of fasted and refed mice.

(B) Immunofluorescent staining of FoxO1 in primary hepatocytes isolated from control and LiRiKO mice.

(C) Glucose production from primary hepatocytes. Hepatocytes were incubated in glucose production media in the presence or absence of insulin, and glucose release into the media was measured after 12 hr incubation. Results are representative of three independent experiments. Results are normalized to the level of control without insulin.

(D) mRNA levels of G6Pase, PEPCK, and PGC-1α in the liver of control and LiRiKO mice after overnight fasting and after 4 hr of refeeding. Results are normalized to the levels of fasted control mice.

and converts glucose to glucose-6-phosphate (G6P). G6P is metabolized through glycolysis, glycogen synthesis, and via the pentose phosphate shunt. In the liver of LiRiKO mice, GK was markedly decreased, as determined by the levels of GK mRNA, protein, and activity (Figures 4F–4H). This correlated with decreased ChREBP expression and nuclear translocation (Figures 4F and S3D), and low expression of a ChREBP target gene (L-pyruvate kinase) in LiRiKO liver (Figure 4F). Furthermore, insulin-induced glycogen synthesis was also significantly decreased in LiRiKO hepatocytes (Figure S3E). The reduced glycogen synthesis in LiRiKO liver could be explained not only by reduced glucose uptake, but also by lower glycogen synthase (GS) activity, as indicated by higher GS phosphorylation (Figure S3F). This was consistent with the observed hypophosphorylation of GSK3, a kinase responsible for inhibitory phosphorylation of GS, in LiRiKO liver (Figure 3B). In contrast, GLUT2 expression in the liver of LiRiKO mice was not changed (Figures S3G and S3H). Taken together, these results indicate that glucose uptake, glycolysis, and glycogen synthesis are impaired in the liver of LiRiKO mice, possibly due to decreased mTORC2-mediated insulin-induced GK expression. Thus, constitutive gluconeogenesis and defective glycolysis (and glycogen synthesis) together account for the observed hyperglycemia. Furthermore, the above findings suggest that defective mTORC2-Akt signaling (hepatic insulin resistance) accounts for the hyperglycemia in LiRiKO mice.

Hepatic mTORC2 Is Required for Insulin-Stimulated De Novo Lipid Synthesis

The glycolytic defect described above and the decreased TG levels in the liver of LiRiKO mice suggested that mTORC2 is required for hepatic lipogenesis. Hepatic lipogenesis is induced by insulin via stimulation of lipogenic gene expression. To investigate lipogenesis, we examined both lipogenic gene expression in the liver of refed mice and de novo lipid synthesis in insulin-stimulated primary hepatocytes. mRNA levels of the key lipid synthesis enzymes, including ATP citrate lyase (ACL), acetyl-CoA carboxylase (ACC), fatty acid synthase (FAS), stearoyl-CoA desaturase (SCD1), glycerol-3-phosphate acyl transferase (GPAT), and diacylglycerol acyl transferase (DGAT2), were all significantly decreased in the liver of refed LiRiKO mice (Figure 5A). In addition, mRNA expression of SREBP1c and PPARγ, two transcription factors that activate lipogenic genes, was significantly reduced in LiRiKO liver (Figure 5A). De novo lipid synthesis was also significantly decreased in insulin-stimulated LiRiKO hepatocytes (Figure 5B). Thus, impaired lipid synthesis in the liver of LiRiKO mice is due, at least in part, to decreased expression of lipogenic genes. Conversely, genes related to fatty acid oxidation, such as acyl-CoA oxidase (ACO), carnitine palmitoyltransferase 1 (CPT1), and PPARα, were increased in fasted LiRiKO mice (Figure S5A). This observation together with a trend of increased plasma ketone bodies (β-hydroxybutyrate) (Figure S5B) suggests higher fatty acid oxidation in the liver of LiRiKO mice. Moreover, expression of genes encoding fatty acid uptake, including fatty acid-binding protein 1 (FABP1) and CD36, was significantly decreased in the liver of HFD-fed LiRiKO mice, while expression of genes encoding fatty acid export, including ApoB and MTP, was unaffected (Figure S5C). Taken together, these observations suggest that the mTORC2-deficient liver displays reduced TG levels due to reduced lipogenesis, increased fatty acid oxidation, and reduced uptake.

Postprandial expression of lipogenic genes is mediated mainly by insulin-induced activation of SREBP1c (Shimomura et al., 1999). SREBP1c is synthesized as an ER bound precursor. Upon activation by insulin, SREBP1c moves to the Golgi where it is proteolytically processed into a mature form. The cleaved mature form of SREBP1c translocates into the nucleus, where it upregulates target lipogenic genes (Horton et al., 2002). Insulin-mediated activation of SREBP1c is Akt dependent (Yellaturu et al., 2009a). Interestingly, recent cell culture-based studies suggest that Akt stimulates lipogenesis via mTORC1-dependent SREBP1 activation (Düvel et al., 2010; Li et al., 2010; Peterson et al., 2011; Porstmann et al., 2008; Yecies et al., 2011), whereas mainly in vivo studies suggest that Akt activates SREBP1c and lipogenesis via an mTORC1-independent pathway (Wan et al., 2011; Yecies et al., 2011). To investigate further the role of mTORC2(-Akt) signaling in lipogenesis, we examined SREBP1c activation in the liver of refed LiRiKO mice. In particular, we examined the levels of precursor and mature forms of SREBP1c. The mature nuclear form of SREBP1c was significantly decreased in the liver of refed LiRiKO mice, whereas the precursor form was unchanged (Figure 5C), suggesting that the posttranslational activation of SREBP1c is defective in LiRiKO liver. Expression of the SREBP1c target genes ACC and FAS was reduced (Figures 5A and 5C). Thus, mTORC2 is required for SREBP1c activation and lipogenesis in the liver.

Constitutively Active Akt2 Restores Glucose Flux and Lipogenesis in mTORC2-Deficient Hepatocytes

The above findings suggest that loss of hepatic mTORC2 results in defective Akt activation (Ser473 phosphorylation) which eventually results in impaired metabolic homeostasis. To investigate further whether loss of mTORC2-mediated Akt Ser473 phosphorylation is responsible for the defects in glycolysis and lipogenesis observed in LiRiKO hepatocytes, we determined if a constitutively active Akt is able to suppress these defects. By adenoviral gene transfer, we introduced an Akt2 mutant in which Ser474 (equivalent to Ser473 in Akt1) is mutated to phosphomimetic aspartic acid (Akt2-S474D). This Akt mutant does not require mTORC2 for activation and is thus considered constitutively active. LacZ and wild-type Akt2 (Akt2-WT) were used as controls. Insulin-stimulated phosphorylation of Akt substrates FoxO1 and GSK3β was significantly reduced in

(E) 2-deoxyglucose uptake in primary hepatocytes. Results are representative of three independent experiments.
(F) mRNA expression levels of GK, liver-type pyruvate kinase (PK) and ChREBP in the liver of 4 hr refed mice. Results are normalized to the levels of control mice.
(G) Western blot analysis of GK expression in the liver of ad libitum fed mice.
(H) Enyzmatic activity of GK and hexokinase in the liver of ad libitum fed mice. Results are representative of three independent experiments. Black and dark gray bars represent control, and white and light gray bars represent LiRiKO (n = 6–7 per group for mice). Values are expressed as mean ± SEM. * indicates statistical significance from control, # indicates statistical significance from respective fasted or untreated control. ($^{*,\ \#}p < 0.05$, $^{**,\ \#\#}p < 0.01$, $^{***,\ \#\#\#}p < 0.001$).

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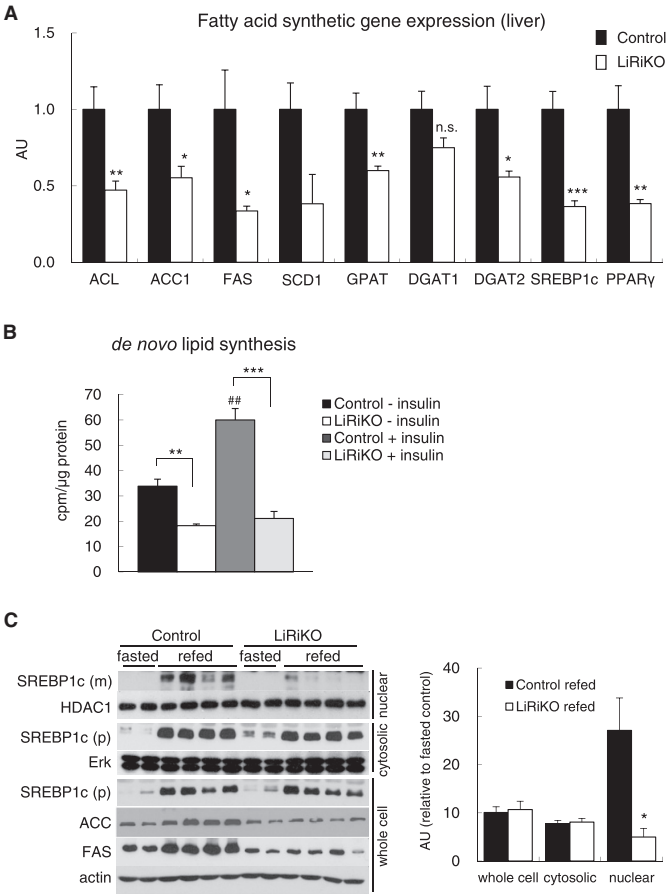

Figure 5. Hepatic mTORC2 Is Required for Insulin-Stimulated De Novo Lipid Synthesis

(A) mRNA levels of genes involved in de novo lipid synthesis including ACL, ACC1, FAS, SCD1, GPAT, DGAT1/2, SREBP1c, and PPARγ, in the liver of mice after 4 hr refeeding. Results are normalized to the levels of control mice. Black bars represent control mice and white bars represent LiRiKO mice (n = 6–7 per group).

(B) De novo lipid synthesis in primary hepatocytes. Serum-starved primary hepatocytes were stimulated with 50 nM insulin for 12 hr. ^{14}C-labeled acetate incorporation into cellular lipids was determined as described in Experimental Procedures. Results are representative of three independent experiments. Black and dark gray bars represent control hepatocytes, and white and light gray bars represent LiRiKO hepatocytes.

(C) SREBP1c processing was analyzed by western blot analysis on nuclear and cytosolic fractions from the liver of mice after 4 hr refeeding. The precursor form of SREBP1c (SREBP1c(p)) was detected in cytosolic fraction and whole cell lysates, and the mature form of SREBP1c (SREBP1c(m)) was detected in nuclear fraction. HDAC1 and Erk were used as marker proteins of nuclear and cytosolic fractions, respectively. Protein levels of SREBP1c target genes (ACC and FAS) were also analyzed using whole-cell lysates. Bar graph to the right represents quantification of band intensities of SREBP1c normalized to its respective internal control. Average value from fasted controls was set to 1. Values are the mean ± SEM, * indicates statistical significance from control, and # indicates statistical significance from respective insulin-untreated control (*, #$p < 0.05$, **, ##$p < 0.01$, ***, ###$p < 0.001$).

lacZ- or Akt2-WT-expressing LiRiKO hepatocytes compared to control hepatocytes (Figure 6A). In contrast, mild overexpression of Akt2-S474D increased insulin-stimulated phosphorylation of FoxO1 and GSK3β in LiRiKO hepatocytes, as in control hepatocytes (Figure 6A). Importantly, restoration of Akt signaling in LiRiKO hepatocytes suppressed the previously observed defects in glucose uptake (Figure 6B) and de novo lipid synthesis (Figure 6C). Furthermore, Akt2-S474D restored expression of GK, SREBP1c, and FAS, in LiRiKO hepatocytes (Figure 6D). Thus, mTORC2-mediated Akt phosphorylation is essential for insulin-induced glycolysis and lipid synthesis in the liver.

Restoration of Glucose Flux Is Not Sufficient to Rescue De Novo Lipid Synthesis in mTORC2-Deficient Hepatocytes

mTORC2-mediated Akt phosphorylation enhances both insulin signaling and glucose flux, which then contribute to activation of lipogenesis (see Introduction and above). To investigate if the defect in lipogensis observed in LiRiKO hepatocytes is due to a defect in glucose flux, we restored glucose flux by overexpressing GK (Figure 7A) and examined if this was sufficient to suppress the lipogenesis defect. Overexpressed GK enhanced glucose uptake to a similar extent in LiRiKO and control hepatocytes (Figure 7B). Consistent with elevated glucose uptake, expression of genes encoding ChREBP, pyruvate kinase, and lipogenic enzymes (FAS, ACC1/2) was significantly increased in GK-overexpressing LiRiKO and control hepatocytes (Figure 7C). However, the defect in de novo lipid synthesis was not suppressed in LiRiKO hepatocytes (Figure 7D). Whereas de novo lipid synthesis was significantly elevated upon GK overexpression, the increase was similar to that observed in control cells (about 2.6-fold), and LiRiKO hepatocytes still displayed only 30% activity compared to control cells (Figure 7D). Thus, glucose flux, although required, is not sufficient to induce lipogenesis. Overexpressed GK did not suppress the defect in Akt Ser473 phosphorylation (Figure 7A) or rescue expression of SREBP1c and its target gene *SCD1* (Figure 7C), indicating that activation of Akt and SREBP1c is essential for lipogenesis in the liver. Taken together, the above findings suggest that mTORC2 activates Akt, which stimulates glycolysis and lipogenesis via GK and SREBP1c (Figure 7E).

DISCUSSION

In this study, we demonstrate that deletion of mTORC2 in the liver (LiRiKO mice) leads to enhanced gluconeogenesis and

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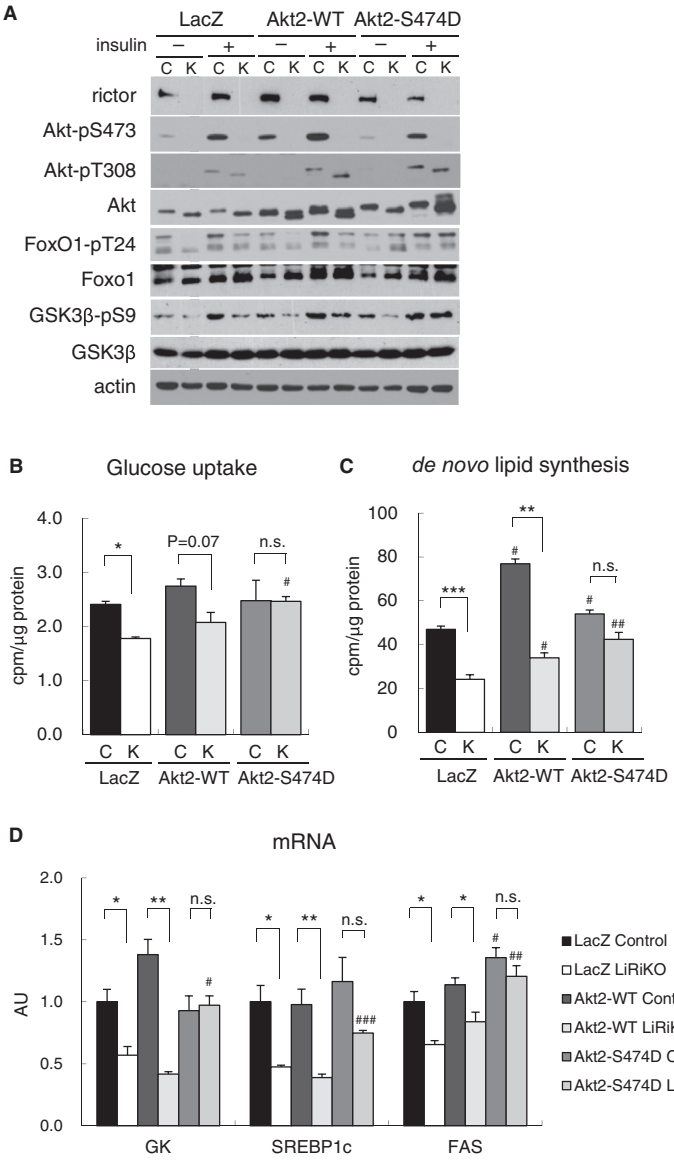

Figure 6. Expression of Constitutively Active Akt2 Restores Glucose Uptake and Lipogenesis in mTORC2-Deficient Hepatocytes

(A) Western blots of control (C) and LiRiKO (K) hepatocytes infected with adenoviruses expressing lacZ, Akt2-WT, and Akt2-S474D. Infected cells were serum starved and stimulated with 50 nM insulin for 30 min.

(B) Glucose uptake by hepatocytes infected with adenoviruses as in (A).

(C) De novo lipid synthesis in hepatocytes infected with adenoviruses as in (A).

(D) mRNA expression levels of GK, SREBP1c, and FAS in hepatocytes infected with adenoviruses as in (A). Results are normalized to the levels of lacZ expressing control hepatocytes.

Values are the mean ± SEM. * indicates statistical significance from control in the same condition and # indicates statistical significance from respective lacZ expressing control ($^{*,\#}p < 0.05$, $^{**,\#\#}p < 0.01$, $^{***,\#\#\#}p < 0.001$).

decreased glycolysis, glycogen synthesis, and lipogenesis in the fed state, despite hyperglycemia and hyperinsulinemia. This suggests that the mTORC2-deficient liver is unable to make the fasted to postprandial transition. In other words, the mTORC2-deficient liver cannot sense satiety. The constitutively active gluconeogenesis was due to loss of both Akt Ser473 phosphorylation and FoxO1 inhibition, and decreased glycolysis was due to reduced GK and glucose uptake. These two defects in hepatic glucose metabolism in turn led to systemic hyperglycemia and hyperinsulinemia. The decreased hepatic lipogenesis was due to reduced SREBP1c activation and low glucose flux. Finally, constitutively active Akt restored both glucose uptake and lipogenesis, whereas overexpression of GK (enhanced glucose flux) failed to restore lipogenesis. Our findings suggest that hepatic mTORC2 controls Akt Ser473 phosphorylation, and thereby GK and SREBP1c, to coordinate glucose and lipid metabolism (Figures 7E and S5).

Another important observation in this study is that LiRiKO mice are phenotypically similar (glucose intolerance, hyperglycemia, hyperinsulinemia, hypolipidemia, and smaller liver size with decreased glycogen content) to many other genetically modified mice that have more severe defects in the hepatic insulin-PI3K-Akt signaling pathway. The mice with more severely defective insulin signaling include liver-specific insulin receptor knockout mice (Biddinger et al., 2008; Michael et al., 2000), single and double hepatic IRS1/2 knockout mice (Dong et al., 2006; Kubota et al., 2008), and mice with deletion of regulatory subunits of PI3K in the liver (Miyake et al., 2002; Taniguchi et al., 2006). Since insulin signaling is completely ablated in these mice, a broad spectrum of downstream targets is affected, including both mTORC1 and mTORC2. In contrast, deletion of mTORC2 results in loss of phosphorylation of the hydrophobic motif, including Akt Ser473 phosphorylation, in a subset of AGC kinase family members, without affecting mTORC1 signaling. The loss of Akt Ser473 phosphorylation accounts for constitutively active FoxO1 and GSK3 and for the loss of

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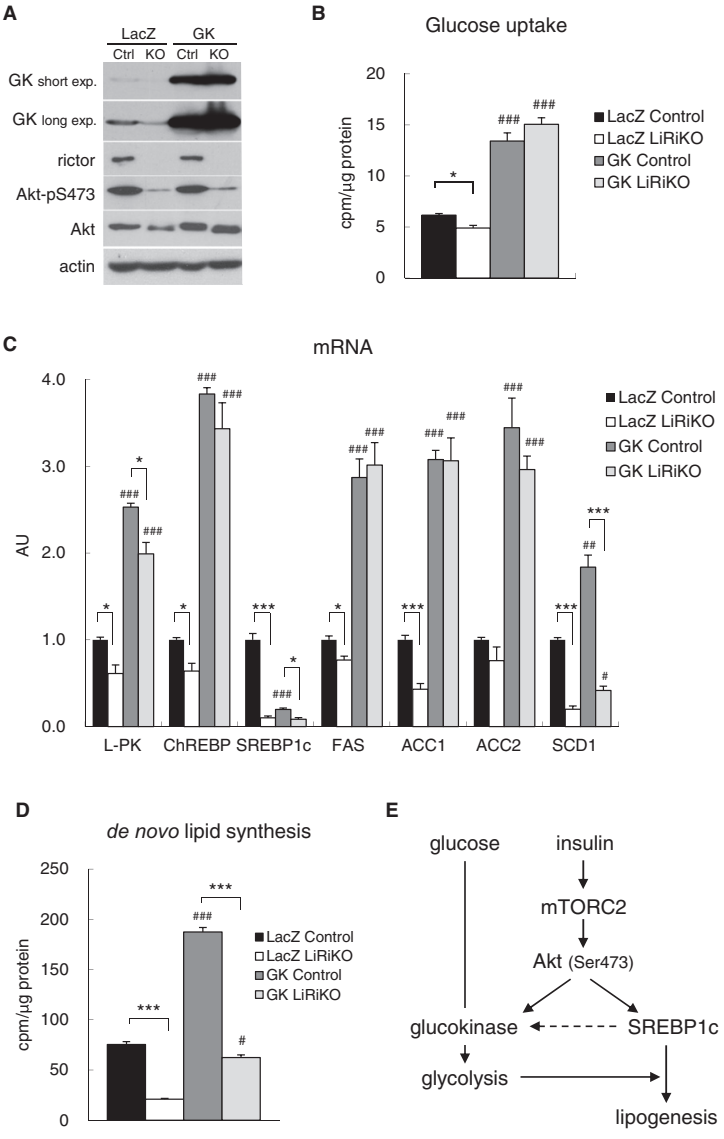

Figure 7. Restoration of Glucose Flux Is Not Sufficient to Rescue De Novo Lipid Synthesis in mTORC2-Deficient Hepatocytes

(A) Western blots of hepatocytes infected with adenoviruses expressing lacZ or GK.

(B) Glucose uptake by hepatocytes infected with indicated adenoviruses.

(C) mRNA expression levels of glycolytic and lipogenic genes. Results are normalized to the levels of lacZ-expressing control hepatocytes.

(D) De novo lipid synthesis in hepatocytes infected with indicated adenoviruses. Black and dark gray bars represent control hepatocytes, white and light gray bars represent LiRiKO hepatocytes. Values are expressed as mean ± SEM of three independent experiments. * indicates statistical significance from control in the same condition and # indicates statistical significance from respective lacZ-expressing control ($^{*,\#}p < 0.05$, $^{**,\#\#}p < 0.01$, $^{***,\#\#\#}p < 0.001$).

(E) Model for the role of mTORC2 in the liver.

SREBP1c and GK activities, and thereby appears to be largely responsible for the phenotype of LiRiKO mice. Thus, our findings reveal a particularly important role for mTORC2 and Akt Ser473 phosphorylation in the hepatic insulin pathway in controlling liver and whole-body metabolism.

Akt is a major regulator of insulin-induced lipogenesis in the liver. Hepatic overexpression of constitutively active Akt or deletion of hepatic PTEN increases TG synthesis (Horie et al., 2004; Ono et al., 2003; Stiles et al., 2004). Conversely, liver-specific deletion of Akt2, the major Akt isoform in the liver, protects against diet- or PTEN loss-induced hepatic steatosis (He et al., 2010; Leavens et al., 2009). Akt promotes lipid synthesis primarily through the activation of SREBP1c, a master transcription factor of lipogenic genes. How does Akt activate SREBP1c? Several cell culture-based studies have suggested that Akt activates SREBP1c and lipogenesis via mTORC1 (Düvel et al., 2010; Li et al., 2010; Porstmann et al., 2008; Yecies et al., 2011). Furthermore, a recent study showed that mTORC1 regulates SREBP by controlling nuclear translocation of lipin1, an inhibitor of SREBP activity (Peterson et al., 2011). However, recent animal-based studies using liver-specific TSC1 or Akt2 knockout mice have shown that mTORC1 activation is not sufficient to stimulate SREBP1c without Akt signaling (Kenerson et al., 2011; Wan et al., 2011; Yecies et al., 2011). Yecies et al. presented evidence that Akt activates SREBP1c via inhibition of expression of Insig2, an anchor protein that retains SREBP1c on the ER membrane (Yellaturu et al., 2009b). Another potential mechanism by which Akt activates SREBP1c is via Akt-mediated phosphorylation and inhibition of GSK3. GSK3 phosphorylates mature SREBP1 and thereby promotes its degradation by the ubiquitin-proteasome pathway (Bengoechea-Alonso and Ericsson, 2009; Kim et al., 2004; Sundqvist et al., 2005). Which of the above mechanism(s) of Akt-mediated activation of SREBP1c is defective in LiRiKO mice? Our finding that SREBP1c activation and lipogenenesis are impaired in LiRiKO liver, where mTORC2 is defective but mTORC1 is active, suggest that Akt signals to SREBP1c in the liver at least in part independently of mTORC1. Also, we observed no significant difference in *Insig2a* expression in the

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liver of refed LiRiKO mice compared to controls (data not shown). However, we did observe a defect in Akt-mediated GSK3 phosphorylation and decreased expression of mature SREBP1c. Thus, constitutively active GSK3 and enhanced degradation of mature SREBP1c may be the mechanism underlying the observed defect in SREBP1c activation in LiRiKO mice.

Though Akt-activated SREBP1c is considered a primary mediator of insulin-stimulated lipogenesis, GK-activated glucose flux also mediates induction of lipogenesis in the liver (Dentin et al., 2005). In liver-specific GK knockout mice, glycolytic and lipogenic genes are not maximally induced upon refeeding, even though SREBP1c activation is normal (Dentin et al., 2004). Moreover, a high carbohydrate diet induces expression of glycolytic and lipogenic genes even in SREBP1c-deficient mice (Liang et al., 2002). GK-dependent and SREBP1c-independent induction of glycolytic (pyruvate kinase) and lipogenic (FAS, ACC) genes appears to be mediated by the transcription factor ChREBP (Uyeda and Repa, 2006). GK is necessary for both expression and activation of ChREBP. Therefore, GK/glucose flux and SREBP1c act synergistically in the stimulation of lipogenesis. In the liver of LiRiKO mice, lipogenesis was impaired due to a decrease in both GK and SREBP1c activity. The decrease in GK expression in the liver of LiRiKO mice is due to a decrease in Akt signaling (the defect is suppressed by activated Akt). GK gene expression is regulated by a number of Akt-dependent transcriptional factors, including FoxO1, HIF-1α/HNF4/p300, SREBP1c, LXRα, and PPARγ (Hirota et al., 2008; Kim et al., 2009;Massa et al., 2011; Roth et al., 2004). Indeed, phosphorylation of FoxO1 and expression of SREBP1c and PPARγ were decreased in the liver of LiRiKO mice. Further studies are required to determine which mTORC2-regulated transcription factor(s) is lacking and thus unable to activate GK expression in LiRiKO mice.

We provide evidence that overexpression of GK in LiRiKO hepatocytes restores expression of glycolytic and lipogenic genes in the absence of Akt Ser473 phosphorylation and SREBP1c activation. However, the level of de novo lipid synthesis induced by GK overexpression in LiRiKO hepatocytes was less than that of control hepatocytes overexpressing GK, despite a similar rate of glucose uptake, suggesting that glucose flux is not sufficient to stimulate lipogenesis. Akt signaling and SREBP1c activation are also required for induction of lipogenesis. Thus, mTORC2 is required for the coordinated regulation, by GK and SREBP1c, of insulin-induced glycolysis and lipogenesis.

In summary, the present study demonstrates the functional importance of hepatic mTORC2 in the regulation of glucose and lipid homeostasis. Hepatic mTORC2 inhibition results in decreased glucose metabolism caused by decreased Akt activity (loss of Akt Ser473 phosphorylation) and subsequently reduced SREBP1c and GK activities. These defects in turn lead to hyperglycemia and hyperinsulinemia. These findings should be taken into account when considering clinical use of emerging mTOR inhibitors that target mTORC2 in addition to mTORC1 (Benjamin et al., 2011). Although detailed safety profiles of mTOR kinase inhibitors remain to be seen, inhibition of mTORC2 may contribute to adverse metabolic effects (Markman et al., 2010; Zhang et al., 2011). Our findings suggest that a defect in hepatic mTORC2 signaling could contribute to metabolic disorders.

EXPERIMENTAL PROCEDURES

A complete version of Experimental Procedures is shown in Supplemental Information.

Animals

rictor$^{fl/fl}$ mice were generated as described previously (Cybulski et al., 2009). Liver-specific rictor knockout mice were generated by crossing *rictor*$^{fl/fl}$ mice with transgenic mice expressing Cre recombinase under the control of the hepatocyte specific *albumin* promoter (C57BL/6-Tg(*Alb-cre*)21Mgn/J; Jackson Laboratory). The resultant *rictor*$^{fl/+}$ *Alb-Cre*$^{Tg/0}$ progeny were crossed with *rictor*$^{fl/fl}$ mice to obtain tissue-specific knockout mice (*rictor*$^{fl/fl}$ *Alb-Cre*$^{Tg/0}$), termed liver-specific rictor knockout (LiRiKO) mice. Littermates without the *Cre* gene were used as wild-type control mice (*rictor*$^{fl/fl}$). All experiments were conducted on male mice between 12 to 17 weeks of age, unless otherwise indicated. All experiments were performed in accordance with federal guidelines and were approved by the Kantonales Veterinaeramt of Kanton Basel-Stadt.

Glucose, Insulin, and Pyruvate Tolerance Tests

For glucose tolerance tests (GTT), mice were fasted overnight (16 hr) then administrated 2 g/kg of body weight of glucose by intraperitoneal (i.p.) injection. For insulin tolerance tests (ITT), mice were fasted for 6 hr or overnight before i.p. administration of 0.5 or 0.25 unit/kg of body weight of insulin (Actrapid). For pyruvate challenge test, mice were fasted overnight then administrated 2 g/kg of body weight of sodium pyruvate by i.p. injection. Blood glucose concentrations were measured before and after the injection at the indicated time points.

Adenovirus Generation

We generated adenoviruses encoding wild-type Akt2 and a phosphomimetic Akt2-S474D mutant using the ViraPower Adenoviral Gateway Expression System (Invitrogen). In brief, wild-type human Akt2 and a phosphomimetic Akt2-S474D mutant, in which Ser at residue 474 is substituted by Asp, were subcloned by PCR from pFastBac-PKBβ and pFastBac- PKBβ-S474D clones (clones were kind gifts of Brian A. Hemmings, FMI, Switzerland) and cloned into pDONR™221 vectors. Entry clones and the pAd/CMV-DEST vector were recombined to generate expression clones (pAd/CMV-Akt2-WT and pAd/CMV-Akt-S474D). Each expression clone was transfected into HEK293A cells to produce adenoviruses. After several amplifications by infecting HEK293A cells, we determined the titer of each adenovirus. AxCA-GK and AxCA-lacZ adenoviruses were a kind gift of Wataru Ogawa (Kobe University, Japan).

Primary Hepatocyte Isolation and Cultures

Hepatocytes were isolated from 8- to 12-week-old male mice using the standard two-step collagenase perfusion protocol. Adenoviral infection experiments were performed 3 hr after plating at an moi of 1 for Akt2 restoration and moi of 5 for GK restoration. Six hours after infection, cells were washed with PBS and incubated overnight with DMEM (5 mM glucose) containing 100 nM dexamethasone and 1 nM insulin. Experiments were performed the next day. For insulin stimulation, hepatocytes were cultured in high glucose DMEM (25 mM glucose) with 10 or 50 nM insulin for the indicated times.

Western Blotting

Whole-protein extraction from tissue was performed as described (Polak et al., 2008). Nuclear and cytosolic fractions were prepared using the NE-PER nuclear protein extraction kit (Pierce). Lysates from primary hepatocytes were prepared using lysis buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.5 mM EDTA, 1% Triton, protease inhibitor, and phosphatase inhibitor. Forty micrograms of protein were loaded for SDS-PAGE and analyzed with the indicated antibodies. Immunoblots were performed using the antibodies against the following proteins: rictor, Akt, Akt (pThr308), Akt (pSer473), GSK3α/β, GSK3α/β (pSer9/21), FoxO1, FoxO1/3a (pThr24/32), IRβ/IGFRβ, IRβ/IGFRβ (pTyr1146/1131), S6K1, S6K1 (pThr389), S6, S6 (pSer235/236), 4EBP, 4EBP (pThr37/46), ACC, FAS, Erk, and HDAC (all from Cell Signaling), glucokinase and SREBP1c (Santa Cruz), ChREBP (Novus

Biologicals), Glut2 (a kind gift from Bernard Thorens, University of Lausanne, Switzerland), and actin (Chemicon).

RNA Isolation and Quantitative PCR

RNA isolation and cDNA synthesis were performed as described (Polak et al., 2008). qPCR was performed using the power SYBR green mix (Applied Biosystems) and quantitated using Applied Biosystems StepOnePlus Real-Time PCR Systems (Applied Biosystems). Duplicate runs of each sample were normalized to 16S rRNA (liver) or cyclophilin (hepatocytes) to determine relative expression levels. The sequences for the primer pairs used in this study are listed in Table S1.

Glucose Production Assay

Glucose production assay was performed following Yoon et al. (2001) with modifications. Briefly, primary hepatocytes were cultured in DMEM (5 mM glucose) containing 100 nM dexamethasone in the presence or absence of 50 nM insulin for 16 hr. Cells were washed three times with glucose-free DMEM without phenol red and then incubated with glucose production solution containing 2 mM sodium pyruvate, 20 mM lactate, 100 nM dexamethasone, and 1 mM 8-bromo-cAMP with or without 100 nM insulin. After 12 hr of incubation, glucose concentration in the media was measured using a glucose oxidase kit (Sigma), normalized to the protein amount of the cells. The assay was done in triplicate and values were expressed as arbitrary units relative to control hepatocytes without insulin.

Measurement of Glucose Uptake

Glucose uptake by primary hepatocytes was analyzed following Yang et al. (2002) with modifications. Briefly, after 12 hr serum starvation, cells were cultured in DMEM containing 25 mM glucose, 100 nM dexamethasone, and 10 nM (in the Akt2 restoration experiment) or 50 nM (in the GK restoration experiment) insulin for 12 hr. To measure the rate of glucose uptake, cells were washed three times with PBS and then incubated for 3 hr in DMEM containing 1 μCi/ml 2-Deoxy-2[1,2-^{3}H]glucose (PerkinElmer Life Sciences). The cells were washed three times with ice-cold PBS and solubilized in 1% SDS. The radioactivity of an aliquot was determined in a scintillation counter. Each assay was done in triplicate, and results were expressed as cpm in the cell lysate/mg of protein.

De Novo Lipid Synthesis Measurement

For de novo lipogenesis analysis, primary hepatocytes were precultured in the same way as for the glucose uptake experiment. Then cells were incubated in culture medium containing 0.5 mM sodium acetate and 0.8 μCi/ml [1-^{14}C] acetic acid (Amersham) for 2 hr. After washing with PBS, the cells were lyzed in 0.5 M KOH. The lysates were neutralized with 6% Na_2SO_4, then the lipids were extracted using sequential extraction with chloroform:methanol (2:1) and chloroform:methanol:H_2O (10:10:3). Radioactivity in lipid-containing phase was measured using scintillation counter. Each assay was done in triplicate, and results were expressed as cpm in the original cell lysate/mg of protein.

Statistical analysis

Student's unpaired t test was used to determine differences among two groups. Significance was judged when p value is less than 0.05. Error bars in figures represent standard error of the mean (SEM).

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures, one table, Supplemental Experimental Procedures, and Supplemental References and can be found with this article online at doi:10.1016/j.cmet.2012.03.015.

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Charles Betz

34, St. Johanns-Ring
Basel 4056, Switzerland
Phone: 0041 79 723 5236
✉ *charles@betz.lu*

Age 28

Nationality Luxembourg

Education

2008 – 2012 **PhD Studies, Leslie Misrock Fellowship**, *University of Basel, Biozentrum*, Growth & Development, Research Group Prof. Michael N. Hall.
Project entitled: mTOR complex 2-Akt signaling is physically and functionally at MAM.
PhD Advisory Committee: Prof. Michael N. Hall, Prof. Nancy Hynes, Prof. Markus Rüegg
Graduation expected 2012

2006 – 2007 **Master of Science in Molecular Biology**, *University of Basel, Institute for Medical Microbiology*, Experimental Oncology, Research Group Prof. Christoph Moroni.
Project entitled: The role of the mTOR pathway in mast cell transformation.
Supervisors: Prof. Christoph Moroni (IMM) and Prof. Michael Hall (Biozentrum).
Grade: 5.8/6.0

2003 – 2006 **Bachelor of Science in Biology, Major in Molecular Biology**, *University of Basel, Biozentrum*, Klingelbergstrasse 50, Basel.
Grade: 5.2/6.0

1996 – 2003 **Secondary School, Section C (Latin, Mathematics, Natural Sciences)**, *Lycee Michel Rodange*, 30, Boulevard Pierre Dupong, Luxembourg.
Grade: Very good (highest grade)

Languages

- English (Very good)
- German (Very good)
- Japanese (Basic)
- French (Very good)
- Luxembourgish (Native)

Work experience

January 2008 – July 2008 **Fulbright Fellowship: Harvard Medical School**, *Brigham and Women's Hospital*, Translational Medicine, 4 Blackfan Circle, Boston, MA, 02115, USA.
Research internship with Prof. David Kwiatkowski, working on mTOR signalling and the TSC tumor suppressor in mice and cell lines. Sponsored by the Fulbright Commission for Educational Exchange between the US and Europe.

November 2007 – December 2007 **Lab Technician: IMM (Institute for Medical Microbiology)**, *Petersplatz 10, CH-4003 Basel, Switzerland*.
Research on the mTOR pathway in an *in vitro* model for experimental oncology.

September 2005 – October 2005

Lab Assistant: LBMCC (Laboratory of Molecular and Cellular Biology of Cancer), *Hopital Kirchberg*, 9, rue Edward Steichen L-2540 Luxembourg.
Studies on the influence of TNF-α on the expression of EpoR.

Further experience

• Swiss animal course LTK Module 1, 2009.

• Biozentrum PhD Student Committee, Member 2009-present. Responsible for the organization of the yearly PhD Student scientific retreats, editing and typesetting of the retreat abstract books, administrator of the PhD Student Website.

• Unterrichtskommission Phil Nat Faculty, University of Basel, Member 2009-present.

• Advanced computer experience in writing and publishing (Microsoft Word, LaTeX), design (Adobe Illustrator, Adobe Photoshop), programming (HTML and PHP), managing and setting-up of small networks, web servers and administration of websites.

Presentations and Publications

• Poster presentation, USGEB Meeting March 2007, Title: Hematopoietic transformants addicted to the TOR pathway and hypersensitive to rapamycin following loss-of-function or downregulation of TSC2; Betz, Colombi and Moroni, 2007.

• Poster presentation, mTOR signalling in health and disease, London, November 2010, Title: mTORC2 is functionally associated with the ribosome; Betz, Zinzalla, Stracka and Hall, 2010.

• Figure contribution, Owusu-Apenten, R. (2009). Bioactive Peptides. Applications for Improving Nutrition and Health (cover; p163). CRC.

• Oral presentation, Oncology Meeting, June 2007, DKBW, Title: The role of TSC2 and mTOR in mast cell transformation
• Oral presentation, Researchers Abroad, November 2008, University of Luxembourg, Title: Mast cells, mTOR and cancer

• Publication, K Pollizzi, I Malinowska-Kolodziej, C Doughty, **C Betz**, J Ma, J Goto, D Kwiatkowski. A hypomorphic allele of Tsc2 highlights the role of TSC1/TSC2 in signaling to AKT and models mild human TSC2 alleles. *Human molecular genetics* (2009) **18**(13):2378-2387; doi:10.1093/hmg/ddp176

• Publication, M Colombi, KD Molle, D Benjamin, K Rattenbacher-Kiser, C Schaefer, **C Betz**, A Thiemeyer, U Regenass, MN Hall and C Moroni. Genome-wide shRNA screen reveals increased mitochondrial dependence upon mTORC2 addiction. *Oncogene* (2010) **30**(13), 1551-1565. doi:10.1038/onc.2010.539

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• Publication, **C Betz**, D Stracka, C Prescianotto-Baschong, M Frieden, Nicolas Demuarex, MN Hall. mTORC2-Akt signaling is physically and functionally at MAM. Manuscript submitted to *Molecular Cell* (2012)

Awards and Honors

• Fulbright Fellowship, 2008, Fulbright Commission for Educational Exchange between the US and Europe
• Bourse Formation Recherche, 2008-2009, Fonds National de la Recherche, Luxembourg
• Leslie Misrock PhD Fellowship, 2008-2012, Leslie Misrock Foundation, Basel
• Poster Prize for best scientific poster, 2nd place, 2009, Biozentrum PhD Retreat
• Student travel grant, 2010, Biochemical Society, UK
• Poster Prize for best scientific poster, 3rd place, 2010, Biozentrum PhD Retreat
• Chevalier du Laurier d'Or, 2010, awarded by the FNEL, Luxembourg for meritorious actions in favor of scoutism
• RNA 2011 Fellowship award, RNA Society, USA
• Poster Prize for best scientific poster, 1st place, 2012, Biozentrum PhD Retreat

Extracurricular activities

Scouting	Leader in the scout group Les Aigles, Luxembourg, since 2000. Organization of several international summer camps, administration of the homepage and more.
Photography	One of my most passionate hobbies is photography. I have participated in a number of national and international exhibitions. In April 2008, one of my pictures was selected for the final round and exhibited at the World Photography Awards in Cannes, France.
Other interests	Other interests include traveling, cooking, cinematography and Jazz music.

Referees

• Prof. Dr. Michael Hall, Biozentrum
Universitaet Basel, Klingelbergstrasse 50/70 CH-4056 Basel Switzerland
Phone: 0041 61 267 21 50, Fax: 0041 61 267 21 49
Email: m.hall@unibas.ch

• Prof. Dr. Christoph Moroni, Biozentrum
Universitaet Basel, Klingelbergstrasse 50/70 CH-4056 Basel Switzerland
Phone: 0041 61 267 16 24
Email: christoph.moroni@unibas.ch

• Prof. Dr. Nancy Hynes, Friedrich Miescher Institute for Biomedical Research
Maulbeerstrasse 66 CH-4058 Basel, Switzerland
Phone: 0041 61 697 8107, Fax: 0041 61 697 3976
Email: Nancy.Hynes@fmi.ch

• Prof. Dr. David Kwiatkowski, Harvard Medical School
Brigham and Women's Hospital, 1 Blackfan Circle, MA 02115, Boston, USA
Phone: 001 617 355 9005, Fax: 001 617 355 9016
Email: dk@rics.bwh.harvard.edu

Ass ee vlaicht vun iech Häre̲n,
Dien d' Saach méi klor gesait?
Ech wëll nun alles wëssen,
An och wou d' Wourecht lait.

Et ginn duerch d' Welt zwéi Weën:
Sief Wollef oder Schof!
E gudde Fuuss ass béides
Als echte Philosoph.

— **Michel Rodange**
Renert oder de Fuuss am Frack an a Ma'nsgrësst (1872)

COLOPHON

This document was typeset using the typographical look-and-feel `classicthesis` developed by André Miede. The style was inspired by Robert Bringhurst's seminal book on typography *"The Elements of Typographic Style"*. `classicthesis` is available for both LaTeX and LyX:

http://code.google.com/p/classicthesis/

Final Version as of October 3, 2012 (`classicthesis` version 4.0).